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THE ROLE OF THE INTERACTION OF THE INFLUENZA B VIRUS NS1 PROTEIN WITH THE CELLULAR BRD2 PROTEIN

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by

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Dedication

I would like dedicate this dissertation to everybody that helped and worried me during my Ph D course. Especially, my parents and parents-in-law who always supported and encouraged me and my lovely family: my wife, Su Jin Kwon, and two daughters, Eun Su Park and Erin Park who always stay with me and make me happy.

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My family, Su Jin, Eun Su, and Erin, you are really good friends and I always smile in any situation because of you. I love you all!

THE ROLE OF THE INTERACTION OF THE INFLUENZA B VIRUS NS1 PROTEIN WITH THE CELLULAR BRD2 PROTEIN

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Influenza B virus is a major human pathogen causing highly contagious respiratory disease. It accounts for approximately ~30% of influenza virus infection per year. The effector domain of the NS1 protein of influenza B virus (NS1B protein), encompassing the carboxy terminal two thirds of the protein, suppresses interferon-β (IFN-β) synthesis in virus-infected cells by unknown mechanism(s). The induced IFN-β mediates innate immunity. To elucidate the mechanism by which the NS1B effector domain suppresses the production of IFN-β, we identified cellular proteins that interact with the NS1B effector domain. Two approaches were used. The approach that succeeded employed the transfection into cells of plasmids expressing the NS1B effector domain containing two affinity tags. After double affinity purification, co-purified cellular proteins were identified by mass spectrometry. We identified Brd2 as a cellular protein that interacts with the NS1B protein. We established that Brd2 specifically binds to the NS1B effector domain *in vitro*, *in vivo*, and in virus-infected cells. Serial mutagenesis experiments showed the phenylalanine at position 171 (F171) of the NS1B protein is

essential for Brd2 binding. To determine the function of the interaction of Brd2 with the NS1B protein, we generated a recombinant virus encoding an NS1B protein in which F at position 171 was replaced by an alanine. The F171A mutant virus was attenuated, and unlike the wild-type virus, induced the synthesis of IFN-β mRNA. IRF3, a key transcription factor for transcription of the IFN-β gene, was activated in mutant virus-infected cells, but not in wild-type virus-infected cells. Transfection assays implicated the activation of the TBK1 kinase as the step in IRF3 activation that is induced in mutant virus-infected cells. We interpreted these results as showing that Brd2 binding to the NS1B protein is required for suppressing IRF3 activation and IFN-β induction. Attempts at further confirmation by depletion of endogenous Brd2 using RNA interference were not successful because of inefficient knock-down efficiency and nonspecific IFN-β induction. A further complication is that another bromodomain protein, Brd4, interacts with the NS1B protein and could compensate for depletion of Brd2.

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CHAPTER ONE

INTRODUCTION

1. Influenza virus

Influenza virus is a major human pathogen that causes highly contagious respiratory disease. It is associated with mortality especially in infants, old people, and in people with chronic diseases. Typically, influenza virus causes about 200,000 hospitalizations and 36,000 deaths in an epidemic season in the United States. When a new strain appears by antigenic shift, it usually causes increased deaths worldwide, as was the case in 1918, 1957, and 1968. The pandemic influenza virus in 1918 killed up to 50 million people worldwide. The H5N1 virus ("bird flu") has been considered a potential pandemic virus. In addition, the recently emerged H1N1 virus ("swine flu") has become a pandemic virus, although it causes only a relatively moderate disease.

1.1. Virion RNAs and viral proteins encoded from virion RNAs (Figure 1.1)

Influenza virus is a genus of *Orthomyxoviridae* which is characterized by the negative sense, segmented, single-stranded RNAs. Influenza viral genome is composed by 7 or 8 RNA segments. Each segment encodes one or two proteins. Three large segments encode PA, PB1, and PB2 protein and they form an RNA dependent RNA polymerase complex. The viral polymerase complex is responsible for the synthesis of viral mRNA and the replication of viral genomic RNAs in host nucleus. Virion RNAs (vRNAs) are used as templates for the synthesis of viral mRNAs. Viral gene transcription is initiated by a "cap snatching" mechanism whereby the viral polymerase cleaves 10 to 13 base fragments from cellular capped (m^7GpppNm-containing) pre-mRNAs and uses them as primers. Transcription of the vRNAs continues until the polymerase reaches 5 to 7 uridine (U) sequence 15-22 bases from the 5'ends of the vRNAs, at which reiterative copying of the stretch of U residues results in a poly A tail. A two-step process of viral RNA synthesis generates virion RNAs. Full-length copies of vRNAs, called complementary RNAs (cRNA), are synthesized, which are copied to form vRNAs. These two steps are initiated without a primer, unlike the initiation of viral mRNA synthesis. The viral nucleocapsid protein (NP) is reported as a factor directs the viral polymerase to produce the unprimed cRNA or vRNA by directly interacting with the viral polymerase complex (Newcomb et al. 2009). The polymerase complexes are incorporated into viral particles bound to the end of the vRNAs.

Three medium size segments encode nucleocapsid (NP), neuraminidase (NA), and hemmagglutinin (HA). Two glycoproteins, NA and HA, are inserted on the viral particles like spikes and are the antigenic parts. HA associates with N-acetylneuraminic

acid (sialic acid) moieties of membrane proteins on the target cell surfaces and mediates viral attachment. NA facilitates viral particle release from infected cell surfaces by catalyzing the cleavage of sialic acids from the HA proteins of newly formed virions. NP forms ribonucleoprotein complex (RNP) with vRNAs by binding vRNAs at regular intervals.

The remaining two small vRNAs are designated as M and NS segment. Each segment encodes two proteins by splicing mechanism from collinear mRNAs; M1 and M2 from M segment and NS1 and NS2 from NS segment. M1 is a matrix protein which underlies the viral lipid bilayer membrane, interacts with viral RNP and cytoplasmic tail of surface proteins. M2 is an ion channel protein which modulates the endosomal pH containing viral particle in the virus infected cells and results in uncoating of viral RNPs during viral entry step. NS1 is a multifunctional protein which blocks various cellular antiviral activities and exceptionally expressed in infected cells but not incorporated in viral particles. We will discuss more in later parts. NS2 mediates the nuclear export of viral RNPs so it is called as nuclear export protein (NEP).

1. 2. The similarity and difference between influenza B virus and influenza A virus

Influenza virus is divided into types A, B, and C, based on the antigenic differences of the NP and M proteins. Influenza A and B virus have a similar structure. Influenza B virus has 8 segmented RNA genome like influenza A virus. Most RNA segments encode functionally similar proteins. There are minor differences between two

viruses (Figure 1.1 A and B). Influenza A virus encodes PB1-F2 protein by alternative reading frame from the PB1 segment. The PB1-F2 protein is a mitochondrial protein and sensitizes the virus infected cells to apoptotic signals (Zamarin et al. 2005). As opposed to influenza A virus, influenza B virus encodes two extra proteins. Influenza B virus NA segment encodes NB protein via a bicistronic mRNA by using overlapping reading frame. In addition, BM2 protein is translated from second open reading frame (ORF) of a bicistronic mRNA encoded from the M segment instead of the M2 protein of influenza A virus that is encoded by alternative splicing mechanism. The NB protein is an integral glycoprotein that is expressed on the infected cell membrane abundantly and is incorporated into virions. The function of the NB protein remains unclear. The BM2 protein conducts proton into the endosome and is essential for uncoating viral RNPs like the influenza A viral M2 proteins. However, the BM2 is not blocked by amantadine, the inhibitor of the influenza A viral M2 proteins. This result explains the resistance of influenza B virus against amantadine. In addition, as will be discussed later, the functions of the NS1 proteins are different.

Only influenza A virus and influenza B virus are responsible for circulating epidemics in the world. Influenza B viruses account for 30 ~ 40% of seasonal epidemics caused by influenza virus in the United States during last 9 seasons except for 2 seasons (Figure 1.2). Influenza B virus is an almost exclusive human virus, although it has been isolated from seals (Osterhaus et al. 2000). Only two HA subtypes and one NA subtype have been found in influenza B viruses. In contrast, influenza A virus has a wide variety of hosts including birds and mammals and has 16 HA and 9 NA subtypes. There has no

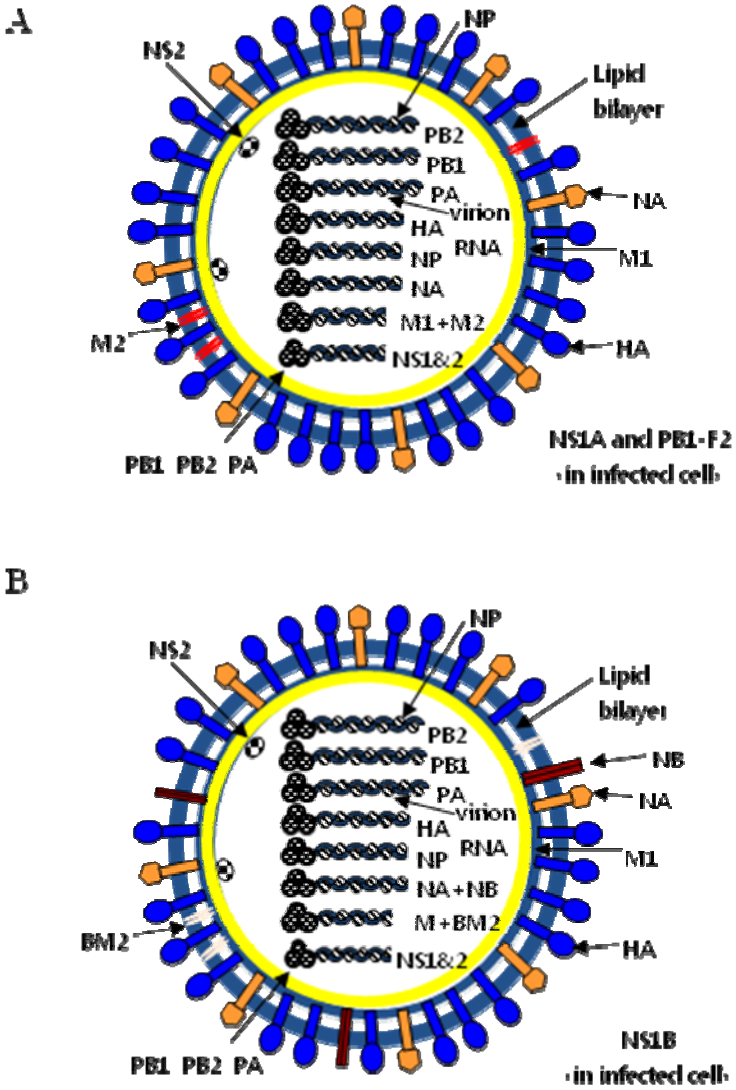

Figure 1.1 Structure of influenza A and B viruses Two diagrams show the structure of influenza A virus **(A)** or influenza B virus **(B)**. Eight segmented negative sense single-stranded vRNAs and the viral proteins encoded from the vRNAs are indicated in the diagrams. The NS1 proteins and the PB1-F2 protein are expressed in viral infected cells but not incorporated into viral particles. Modified from Noah & Krug (2005)

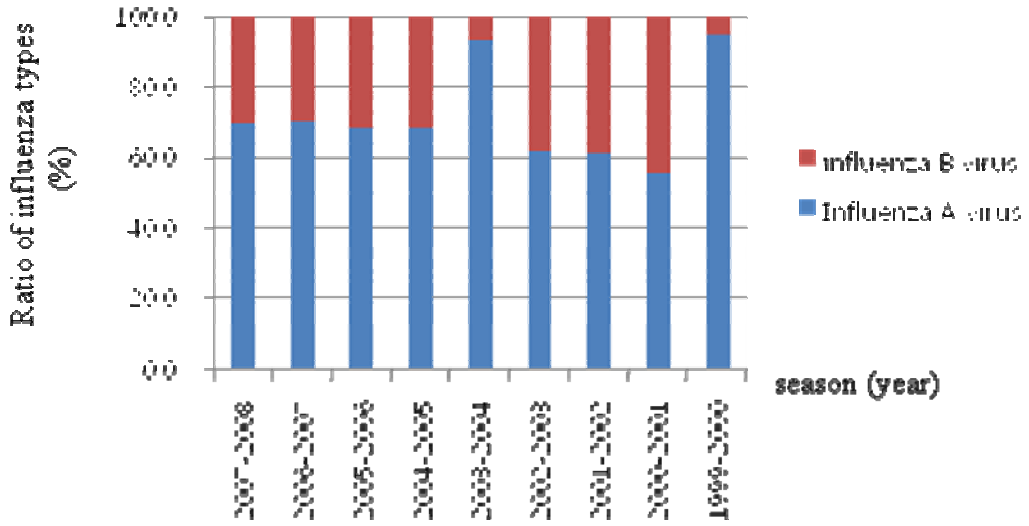

Figure 1.2 Percentage of influenza virus types during last 9 seasons Each bar indicates the percentage of the circulating influenza A virus (blue) or influenza B virus (red) in the USA, collected by the Centers for Disease Control (CDC) from U. S. laboratories. Data from CDC website (http://www.cdc.gov/flu/weekly/fluactivity.htm)

pandemics caused by influenza B viruses been reported because of its narrow host range and limited subtypes of HA and NA genes although pandemics have been caused by influenza A viruses.

2. Influenza virus nonstructural protein 1 (NS1)

NS1 is a multifunctional protein, encoded by the influenza virus NS segment. Structurally, NS1 protein is divided into two domains: the RNA binding domain (RBD) comprising the 73 amino terminal amino acids of influenza A virus NS1 protein (NS1A) and 93 amino terminal amino acids of influenza B virus NS1 protein (NS1B). The effector domain encompasses the rest of the NS1A and NS1B proteins (Figure 1.3 A). The RBDs of the NS1A/NS1B proteins are similar in structure and share double-stranded RNA (dsRNA)-binding functions. In contrast, the effector domain of the NS1A protein binds several proteins that do not bind to the NS1B effector domain, indicating that the functions of these two effector domains differ.

2. 1. The RBDs of NS1A and NS1B

The RBDs of NS1A and NS1B have similar structures (Chien et al. 1997; Liu et al. 1997; Yin et al. 2007), (Figure 1.3 B). They fold as a unique symmetric six-helical

dimer structure. Besides, the double stranded RNA (dsRNA)-binding surface consists of highly conserved regions of basic and hydrophilic residues complementary to the polyphosphate backbone conformation of A-form dsRNA as a major target. The arginines at amino acid 50 (R50) and 53 (R53) of NS1B are essential for the NS1B RBD-dsRNA interaction, and the arginine at amino acid 38 (R38) and the lysine at amino acid 41 (K41) of NS1A are essential. The primary target is A-form dsRNA without sequence-specificity (Wang and Krug 1996). The *in vivo* function of the dsRNA-binding activity of the RBD of NS1B protein in the influenza B virus-infected cells has not yet been determined. The dsRNA-binding activity of the RBD of the NS1A protein has been shown to block the activation of interferon-inducible 2'-5' OAS/RNase L pathway by sequestering dsRNA which can activate the pathway (Min and Krug 2006). A recombinant influenza A virus with the NS1A R38A mutation is more sensitive than wild type influenza A virus to IFN-β treatment, and this enhanced IFN-β susceptibility is relieved by depleting endogenous RNase L with small interfering RNA (siRNA). Furthermore, the susceptibility of mutant virus is partially relieved in RNase L-deficient murine embryonic fibroblasts (MEFs).

2. 2. ISG15

The only known cellular target for the NS1B protein is ISG15, an ubiquitin-like 15kDa protein that is, highly induced by type I interferon and is also induced by activated

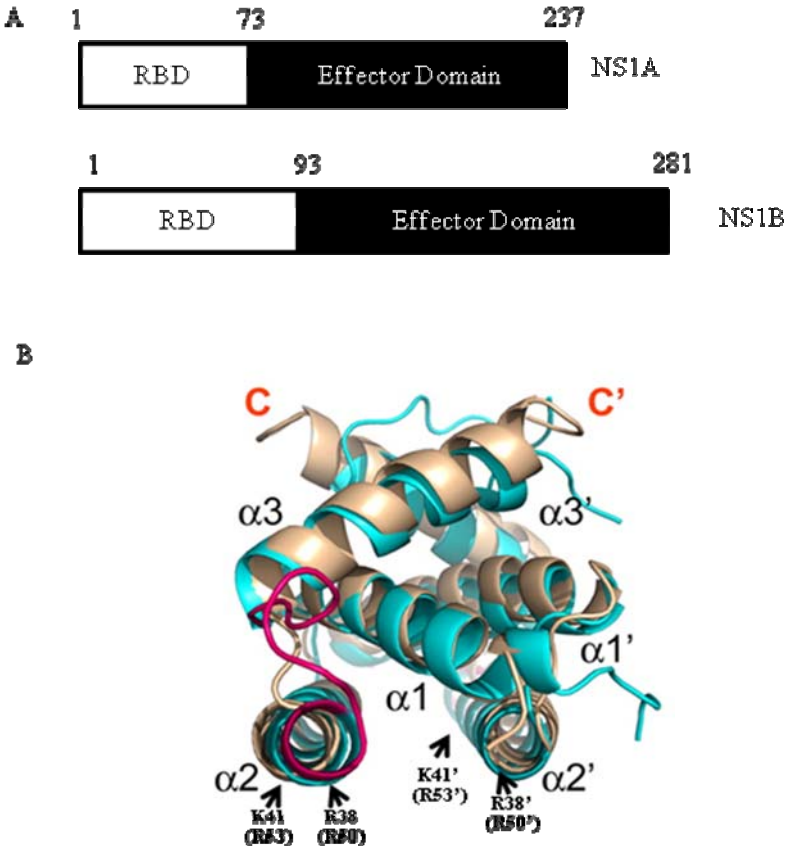

Figure 1.3 Two domains and the structural similarity of RBDs of the NS1A/NS1B proteins A. Simple diagram of two domains of the NS1A/NS1B proteins. The NS1A protein and the NS1B protein are comprised of two domains: N-terminal one third of them are termed the RNA binding domain (RBD) and the rest of the proteins are the effector domain. The numbers on the rectangle are denoted amino acid residues. **B.** The overlap of the crystal structures of NS1A (1-73) (tan) and NS1B (1-103) (blue). Essential amino acid residues for the dsRNA binding on the α2 and α2' helices are indicated by arrow and numbers. The essential residues for NS1B are in the parentheses. Modified from Yin et al. (2007).

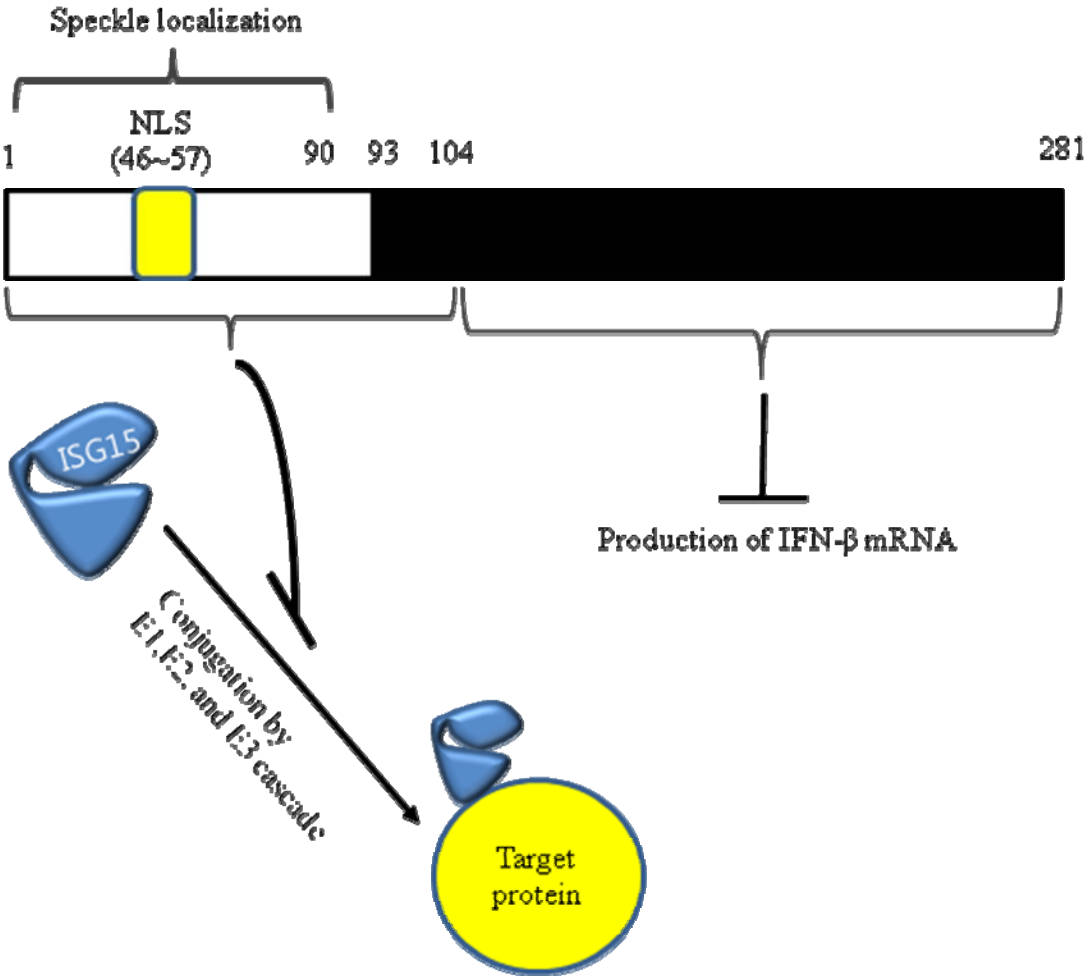

Figure 1.4 Interacting protein and functions of the NS1B protein Diagram shows the NS1 protein of influenza B/Yamanashi/166/98 virus. The amino terminal 93 amino acids (white rectangle) are the RBD. The amino acids 46 to 57 (yellow rectangle) are a nuclear localization signal (NLS) which interacts with importin α family proteins. The amino acids 94 to 281 (black rectangle) are the effector domain which suppresses the IFN-β mRNA synthesis. The N-terminal 90 amino acids mediate the nuclear speckle localization of the NS1B protein. ISG15 (blue) is the only identified interacting protein of the NS1B protein and the N- terminal 104 amino acids are essential for the interaction. The NS1B protein blocks the conjugation of ISG15 to its targets (yellow circle) via its direct interaction with ISG15 in infected cells. The numbers above the rectangle are denoted amino acid residues of the NS1B protein.

IRF3. ISG15 is conjugated to its targets through isopeptide bond formation by the enzymatic cascade involving Ube1L (E1), UbcH8 (E2), and Herc5 or EFP (E3) (Yuan and Krug 2001; Zhao et al. 2004; Dastur et al. 2006; Wong et al. 2006; Zou and Zhang 2006) (Figure 1.4). These three enzymes are also induced by IFN-β. An increased susceptibility of $ISG15^{-/-}$ mice or $Ube1L^{-/-}$ mice to influenza A and B viruses confirmed the antiviral function of ISG15 against influenza viruses (Lenschow et al. 2007; Lai et al. 2009). At least 158 ISG15 targets are identified and those targets mediate various cellular events (Zhao et al. 2005). Free ISG15 is secreted and may activate natural killer cells but this function requires more determination (D'Cunha et al. 1996).

However, it has not been shown how ISG15 and/or its conjugation inhibit the influenza A and B virus replication. The NS1B protein binds human ISG15 and inhibits its conjugation (Yuan and Krug 2001). In contrast, the NS1B protein does not bind mouse ISG15 (Sridharan, P., Zhao, C. and Krug, R, M., unpublished experiments), thereby explaining why influenza B virus is inhibited in ISG15+/+ mice.

2. 3. Function of NS1B effector domain (Figure 1.4)

A major function of the effector domain of the NS1B protein is to inhibit the production of interferon β (IFN-β) mRNA. A recombinant influenza B virus encoding only the N-terminal 104 or 110 amino acids of the NS1B protein is attenuated 10- to 100-fold coupled with increased production of IFN-β mRNA

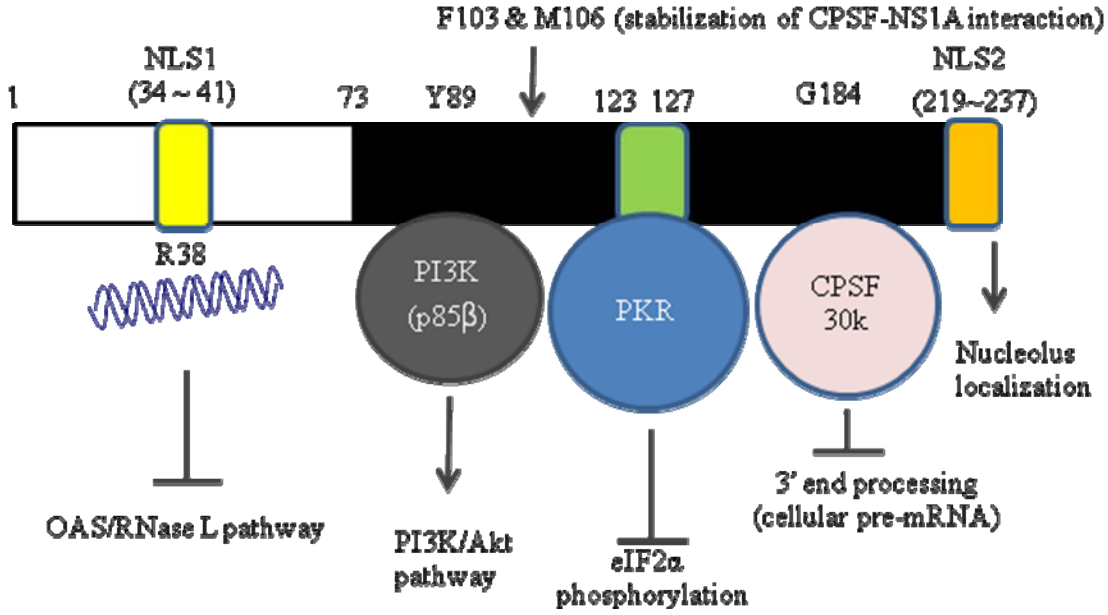

Figure 1.5 Interacting molecules and functions of the NS1A protein The diagram shows the NS1 protein of influenza A/Udorn/72 virus. The amino terminal 73 amino acids (white rectangle) are RBD and the essential amino acid for dsRNA binding is the arginine 38 (R38). The RBD in virus infected cells sequesters dsRNAs (blue helix) which would activate the OAS/RNase L pathway. The tyrosine at position 89 (Y89) is essential to activate the PI3K/Akt pathway by interacting with the 85kDa β subunit of the PI3 kinase (gray circle). The amino acids 123 to 127 are the PKR (blue circle) binding site which interferes with the phosphorylation of eIF2α which inhibits all protein synthesis in infected cells. The glycine at position 184 (G184) is important for forming CPSF (pink circle) binding pocket and, in addition, two amino acids, the phenylalanine at 103 (F103) and the methionine at 106 (M106), stabilize the complex formation. The association of the NS1A protein and CPSF interferes with 3' end processing of cellular pre-mRNAs. The NS1A protein of the Udorn/72 strain has two NLS motifs; one is in RNA binding domain (34 to 41, yellow rectangle) and the other is the C-terminal 19 amino acids (219-237, orange rectangle). NLS2 is essential for the localization to the nucleolus. Numbers are the denoted amino acids of the NS1A protein.

(Dauber et al. 2006; Hai et al. 2008). However, the mechanism(s) by which the effector domain of the NS1B protein inhibits the production of IFN-β mRNA has not been established.

2. 4. Function of NS1A effector domain (Figure 1.5)

As opposed to the NS1B effector domain, extensive studies on the NS1A effector domain have revealed that it interacts with multiple cellular factors to antagonize antiviral responses. The best characterized interacting protein for the NS1A effector domain is the 30kDa component of cleavage and polyadenylation specificity factor (CPSF) (Nemeroff et al. 1998) which binds cellular pre-mRNAs at the AAUAAA sequence upstream of the 3' end cleavage site and is required for the 3'-end processing of pre-mRNAs. Consequently, the NS1A protein inhibits the 3'-end processing of all cellular pre-mRNAs, including antiviral IFN-β pre-mRNA by binding the 30kDa component of CPSF. The glycine at position 184 of NS1A (G184) is essential for forming the CPSF binding pocket, and the phenylalanine at 103 (F103) and the methionine at 106 (M106) are essential for the stabilization of the NS1A-CPSF complex (Das et al. 2008). The NS1B protein does not bind the 30kDa component of CPSF.

Second, NS1A binds the 85 kDa regulatory β subunit (p85β) of phosphatidylinositol-3-kinase (PI3K) and activates PI3K/Akt pathway which is associated with many cellular processes including cell growth, proliferation, and anti-apoptosis. The tyrosine at 89 amino acid of NS1A (Y89) within NS1A effector domain is

required for the interaction (Hale et al. 2006). The activation of PI3K results in the generation of phosphatidylinositol 3, 4, 5-triphosphate from phosphatidylinositol 4, 5-bisphosphate in the membrane. This change recruits Akt, a major effector kinase, which is activated by phosphorylation at threonine 308 and serine 473. By activating the PI3K/Akt pathway, influenza A virus prevents premature induction of apoptosis to achieve the efficient virus replication (Ehrhardt et al. 2007b). However, the activity is not conserved in the NS1B protein (Ehrhardt et al. 2007a).

Third, NS1A interferes with the activation of protein kinase R (PKR) mediated by either dsRNA or cellular PACT protein by binding PKR. Amino acids 123 to 127 of NS1A are required for this binding (Li et al. 2006; Min et al. 2007). Activated PKR would phosphorylate the α subunit of eukaryotic initiation factor 2 (eIF2α), resulting in blocking all protein synthesis including viral protein synthesis.

2. 5. Localization of NS1A and NS1B protein in infected cells

The localization of NS1B is dynamic in virus-infected cells. The NS1B protein localizes in nuclei of infected cells and accumulates mainly in nuclear speckles in early infection phase. However, the protein is relocated in the cytoplasm of the cells in later infection phase. The N-terminal 90 amino acids of the NS1B protein mediate the speckle-localization by unknown mechanism and the amino acids 46 to 57 of NS1B are the nuclear localization signal (NLS) which bind the importin α family proteins (Schneider et al. 2009) (Figure 1.4). The NS1A protein localizes mainly in the nucleus. The NS1A

protein has one or two NLS depending on viral subtypes. The NS1A proteins containing a second NLS can localize in nucleoli the site of ribosomal RNA biogenesis (Figure 1.5). The importin α family proteins are the essential mediator for nuclear localization of NS1A proteins but the mediator for nucleolar localization remains unclear (Melen et al. 2007).

3. Interferons (IFNs)

Interferons (IFNs) regulate innate and/or acquired immunity against pathogens. They bind to specific receptors and induce the transcription of IFN dependent genes. Originally, IFN was discovered as an antiviral agent against influenza virus more than 50 years ago. IFN is the first line of defense against virus infection.

3.1. Types of IFNs and their receptors (Figure 1.6)

IFN is classified into 3 types, named type I to III based on the types of their receptors. Type I IFNs include 13 IFN-α subtypes, IFN-β, IFN-κ, IFN-ε, IFN-ο, IFN-τ and IFN-δ, and bind to the ubiquitous IFN α receptor (IFNAR) that consists of two proteins, IFNARA1 and IFNARA2. Although type I IFNs are produced by almost all types of cells at low level by pathogen infections, IFN-β is mainly secreted by fibroblasts,

and IFN-α and IFN-ω are mainly secreted by hematopoietic cells. Type I IFN induces the transcription of 300 genes (ISGs) (Der et al. 1998). The functions of only a small number of the encoded proteins have been determined.

IFN-γ is the only type II IFN. IFN-γ dimers bind the heterotetrameric IFN-γ receptor complex (IFNGR), made up of two IFNGR1 chains and two IFNGR2 chains. Expression of IFNGR2 chains determines the IFN-γ responsiveness of cells as the expression is tightly regulated and limited to certain cells, whereas IFNGR1 chains are constitutively expressed on all types of cells. The production of IFN-γ is controlled by cytokines secreted by antigen presenting cells (APCs), most notably interleukin (IL)-12 and IL-18. IFN-γ is predominantly secreted from specific types of cells, including natural killer (NK) or activated T cells as a part of innate immunity, and $CD4^+$ or $CD8^+$ cytotoxic T cells as part of adaptive immune response. IFN-γ induces the transcription of 50 genes and modulates various cellular immune responses, including enhancement of NK cell activity, regulation of B cell class switch, and enhancement of antigen presentation by macrophage. IFN-γ activates an antiviral state by inducing PKR, ADAR, and GBP1 & 2 (Schroder et al. 2004).

Type III IFN, which includes three IFN-λ subtypes, binds to heterodimeric receptor complex of IFN-λ receptor 1 (IFNLR)/IL28RA and interleukin-10 receptor 2 (IL-10R2). Type III IFN induces the same repertoire of ISGs as those stimulated by type I IFNs because the downstream effectors are shared between two pathways as described below (Nina Ank 2009) (Figure 1.6).

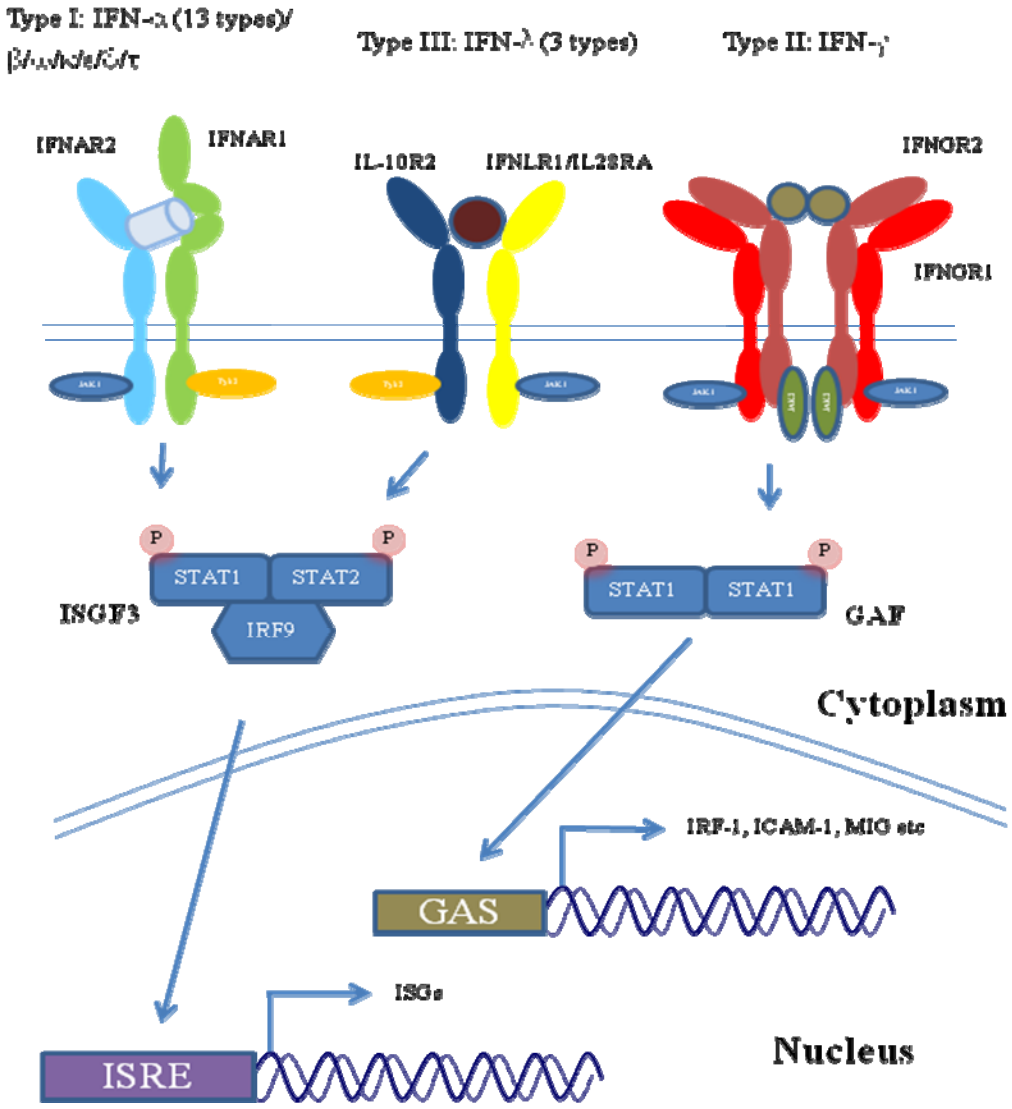

Figure 1.6 Three types of IFNs and their receptor-mediated signaling pathways IFNs are classified into three types, I to III, based on their receptors. Each component of IFN receptors is pre-associated with tyrosine kinases termed as JAK1 (blue oval), Tyk2 (orange oval), or JAK2 (deep green oval). The IFN-ligation on the corresponding receptor activates the associated kinases and then the receptors thereby resulting in recruiting and phosphorylating STAT1 or 2 (blue rectangle). The heterodimer of the phosphorylated STAT1 and 2 associates with IRF9 (blue hexagon) to form ISGF3 under the type I or III IFN-mediated signaling pathway. ISGF3 translocates into the nucleus and binds to a DNA element (ISRE) thereby turning on the transcription of ISG genes. Type II IFN (IFN-γ) stimulates the formation of STAT1 homodimers (GAF) that turns on the transcription of different sets of genes by binding to specific DNA element (GAS). Modified from Borden et al. (2007).

3.2. IFN signaling pathways (Figure 1.6)

The association of IFNs with their IFN receptors turns on signaling cascades by activating pre-associated Janus tyrosine kinases (JAK1 and TYK2 for Type I or Type III IFN receptors: JAK1 and JAK2 for Type II IFN receptors). The activated JAK or TYK is autophosphorylated and then transphosphorylates IFN receptor chains that trigger the recruitment and the phosphorylation of Signal Transducers and Activators of Transcription (STATs). In type I or type III IFN signaling pathways, the heterodimer of phosphorylated STAT1 and STAT2 associates with the IFN regulatory factor 9 (IRF9) and form the IFN-stimulated transcription factor (ISGF3). The ISGF3 localizes in the nucleus and turns on the transcription of ISG genes by binding to the IFN stimulated response element (ISRE) in the promoter of ISG genes. In the case of type II IFN signaling pathways, the homodimer of phosphorylated STAT, designated as the IFN-γ activation factor (GAF), translocates into the nucleus where it binds to the consensus IFN-γ activated site (GAS) element of IFN-γ dependent genes, resulting in their transcription.

3. 3. Type I IFN-dependent ISGs and their functions

Type I IFN treated cells contain a large amount of ISG proteins. Several ISGs have been shown to inhibit viral replication, including ISG15, Mx1, 2'-5' oligo (A) synthetase, and PKR. As already discussed, the growth of various types of viruses are more restricted in $ISG15^{+/+}$ mice than in $ISG15^{-/-}$ mice (Lenschow et al. 2007).

The Mx proteins, which consist of MxA and MxB in humans and Mx1 and Mx2 in mice, are GTPases. The mouse Mx1 protein is localized in the nucleus and inhibits primary transcription catalyzed by the influenza A viral RNPs of the inoculum virus (Krug et al. 1985). The Mx1 protein is specific for influenza virus. Originally, the Mx1 protein was determined as an antiviral protein based on the observation that the inbred mouse strain A2G is resistant to the lethal doses of mouse adapted influenza virus (Lindenmann 1962) and the resistance depends on the Mx1 gene in the A2G strain mouse. The human MxA protein, which is in the cytoplasm, inhibits a later step in influenza virus replication (Pavlovic et al. 1992), and also inhibits other viruses including orthomyxoviruses, paramyxoviruses, rhabdoviruses, togaviruses, and bunyaviruses. The acquired enhanced resistance to viruses of the transgenic mice, constitutively expressing human MxA protein, clearly demonstrates the human MxA protein is an antiviral protein *in vivo* (Pavlovic et al. 1995).

Although the antiviral mechanism of Mx proteins has not been clear, the MxA proteins associate with nucleocapsid proteins of Thogoto virus and trap viral particles in the cytoplasm resulting in blocking the replication of viral genome at early time point (Kochs and Haller 1999). Otherwise, Mx proteins directly interact with viral polymerase components and interfere with the viral replication. For example, the Mx1 protein associates with the influenza viral PB1 protein and blocks the polymerase function (Stranden et al. 1993). Viruses evade the surveillance of Mx proteins. The highly virulent strains of influenza viruses increase their replicative fitness to overcome the IFN signaling (Grimm et al. 2007). Hepatitis B viral precore or core proteins prevent the

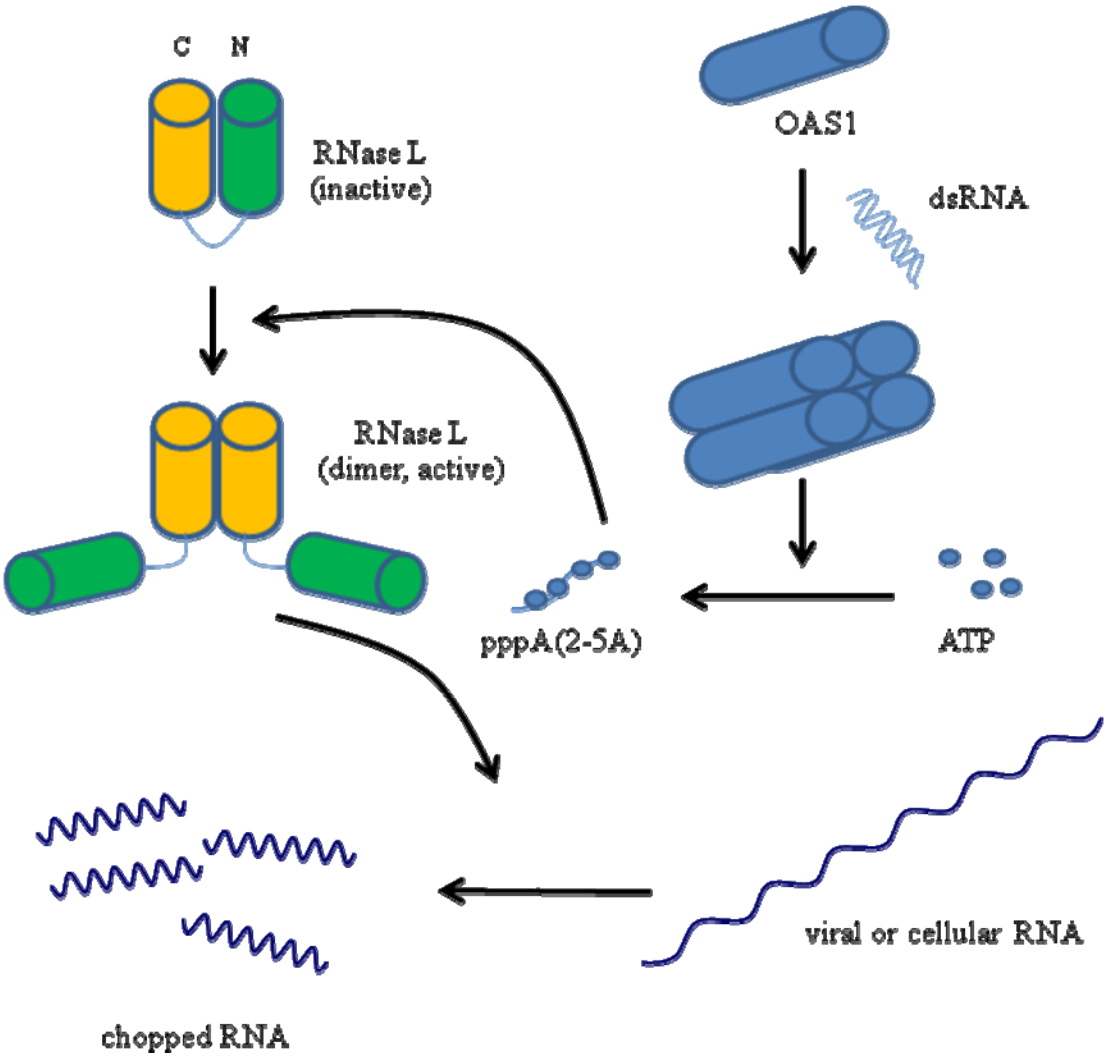

Figure 1.7 Diagram of the antiviral pathway by the OAS1/RNase L pathway OAS1, one of OAS isoforms, is more expressed in cells treated with type I IFN or infected with viruses. The activated OAS1's by binding dsRNA forms tetramers that synthesize tetrameric 2', 5' oligoadenylates (pppA(2-5A)). The association of 2', 5' oligoadenylates with the latent RNase L induces a conformational change to form active RNase L homodimers which chops viral or cellular RNAs. Modified from Sadler and Williams (2008).

transcription of the MxA genes via direct interaction with the MxA promoter (Fernandez et al. 2003).

2', 5'-oligoadenylate synthetase (OAS) is constitutively expressed at a low level and increased by type I IFN treatment. The dsRNA-activated OAS proteins oligomerize, followed by polymerizing ATP to 2'-5' phosphodiester-linked adenosine oligomers that activate constitutively expressed, resting RNase L. The activated RNase L degrades intracellular single-stranded RNAs (ssRNAs) including viral RNAs, cellular mRNAs, and ribosomal RNAs predominantly after UA or UU dinucleotides (Floyd-Smith et al. 1981) (Figure 1.7). In humans, four OAS genes, termed OAS1, OAS2, OAS3, and OASL (OAS-like), have been identified. Each gene encodes one or two proteins by alternative splicing mechanism. Except for p59 OASL protein encoded from the OASL gene, the proteins encoded from the others synthesize different 2-5A oligomers of different lengths (tetrameric 2-5A oligomers for OAS1; trimeric 2-5A oligomers for OAS2; dimeric 2-5A oligomers for OAS3). Because of the functional redundancy among OAS isoforms, the antiviral activity of the OAS/RNase L pathway is mainly studied using RNase L deficient mice. Several viruses are targeted by the IFN induced OAS/RNase L (Silverman 2007).

The influenza A viral NS1 protein blocks the OAS/RNase L pathway by sequestering dsRNAs that would otherwise activate OAS (Min and Krug 2006). The phylogenetically conserved RNA structure of the poliovirus open-reading frame, $3C^{pro}$ proteinase, potently inhibits the activity of RNase L (Han et al. 2007). The OAS/RNase L pathway has additional functions. Because apoptosis is suppressed in RNase $L^{-/-}$ mice, the OAS/RNase L pathway may be involved in an IFN-dependent proapoptotic function that

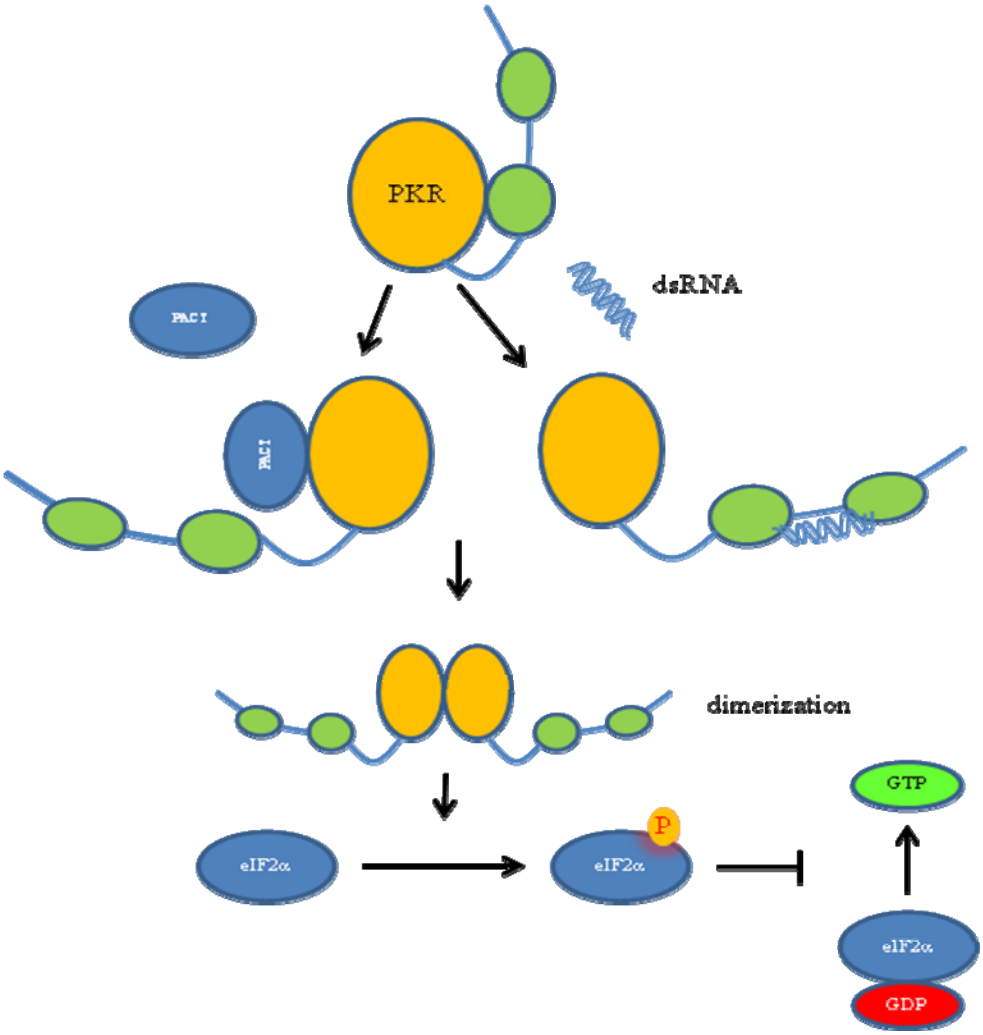

Figure 1.8 Diagram of the antiviral pathway by PKR The amount of PKR is increased by type I IFN. The interaction of dsRNA or PACT with PKR dimerizes and autophosphorylates PKR. The PKR dimer blocks translation by phosphorylating eIF2α, resulting in sequestration of the limiting GDP exchange factor, eIF2β. Modified from Li et al. (2006)

results in the clearing viral infected cells *in vivo* (Zhou et al. 1997). The cellular RNAs degraded by RNase L may activate cytoplasmic RNA recognition receptors such as RIG-I and Mda5 and potentiate innate immunity (Malathi et al. 2007).

PKR is constitutively expressed but is also induced by type I IFN treatment. PKR dimerizes and autophosphorylates after binding dsRNA or the PACT protein, and inhibits the translation by phosphorylating eIF2α, resulting in the sequestration of limiting GDP exchange factor, eIF2β (Figure 1.8). PKR can block the translation in apoptotic cells. PKR can be activated by the active forms of caspase-3, -7, and -8 that cleave the inhibitory N-terminal domain of PKR and generate constitutively active PKR during cellular apoptosis (Saelens et al. 2001). In addition to the phosphorylation of eIF2α, PKR phosphorylates I-κB, resulting in activation of NF-κB signaling (Kumar et al. 1994).

Many viruses evolve mechanisms against the PKR-mediated antiviral responses (Langland et al. 2006). Those are categorized into various mechanisms including the sequestration of dsRNA, production of inhibitory RNAs, inhibition of PKR dimerization via direct association of viral proteins with PKR, expression eIF2α homologues which can act as a pseudosubstrate for PKR, degradation of PKR, and promotion of eIF2α-dephosphorylation by viral proteins.

Another ISG, viperin blocks influenza A virus release from the plasma membrane of the infected cells (Wang et al. 2007).

4. The components of the IFN-β induction pathway in RNA virus-infected

cells and their regulation (Figure 1.9)

The infection of mammalian cells with RNA viruses rapidly provokes an innate immunity, as the first line of defense, producing type I IFNs, especially, IFN-β. The association of pathogen-associated pattern molecules (PAPMs) on the pattern-recognition receptors (PRRs) primes a multistep signaling pathway consisting of various components as described below, resulting in IFN-β production. As an inappropriate or constitutive induction of the pathway might cause harmful effects, such as autoimmune disease, the signaling pathway must be tightly controlled by various mechanisms.

4.1. RIG-I like receptors (RLRs), a cytoplasmic non self RNA recognizer

In mammalian cells, two families of PRRs are responsible for sensing various PAMPs. The inducible, RIG-I like receptor (RLR) family proteins, including RIG-I, Mda5, and LGP2, detect cytoplasmic non-self RNAs in virus-infected cells and Toll like receptor (TLR) family proteins, which comprise 13 members in humans, recognize various PAMP usually reside in intracellular compartments, including endosomes and the endoplasmic reticulum (ER).

In most RNA virus-infected cells, single-stranded RNAs (ssRNAs) with a 5'-terminal triphosphate and dsRNAs are produced and engage RLRs in the cytoplasm and TLR3 in endosome. Although TLR3 can play an important role as non-self dsRNA sensor in cells infected with an RNA virus, in influenza virus-infected lung epithelial cells,

TLR3 is not the major PRR for IFN-β induction (Le Goffic et al. 2007). In the influenza B or A virus-infected fibroblast cells, the viral RNAs (vRNAs) with N-terminal triphosphate are exclusively recognized by RIG-I (Kato et al. 2006; Loo et al. 2008). Interestingly, cytoplasmic dsRNA in cells infected with negative-sense RNA viruses such as influenza viruses are scarcely detected, compared with positive-sense RNA viruses or DNA viruses (Weber et al. 2006).

Both IFN-inducible RIG-I and Mda5 similarly contain two tandem caspase recruitment domains (CARDs) at their N-termini, a single RNA helicase domain in the middle, and a repressor domain (RD) at their C-termini. The mechanism by which RIG-I recognizes RNA is clearer than that by which Mda5 recognizes RNA. RIG-I preferentially detects ssRNA over dsRNA and specifically binds ssRNA containing a 5' triphosphate but not a 5' OH or a 5' methylguanosine cap (Hornung et al. 2006; Pichlmair et al. 2006). In addition, the structure and composition of nucleotides are essential. RIG-I detects better RNA ligands with linear structure, longer than 23 nucleotides but shorter than 2 kb, and poly uridine- or adenosine tracts (Kato et al. 2008; Saito et al. 2008). Binding specific RNA ligands to the C-terminal domain (CTD) of RIG-I, which includes RD of RIG-I, exposes the CARDs, originally blocked by RD, and the RIG-I associates with MAVS, the downstream adaptor protein, through a CARD-CARD interaction (Takahasi et al. 2008). The MAVS, associated with activated RIG-I, trigger the signaling cascade to induce INF-β. The inactive RIG-I splicing variant lacking the region of amino acids 38 to 80 within first CARD or the LGP2 protein which lacks CARD domains interferes with the multimerization of the activated RIG-I, demonstrating the importance

of RIG-I multimerization in the RIG-I dependent induction of IFN-β (Saito et al. 2007; Gack et al. 2008).

Although Mda5 has structure similar to that of RIG-I, they differ in specificity of RNA virus recognition (Kato et al. 2006; Loo et al. 2008) as well as RNA binding specificity (Kato et al. 2008). Mda5 prefers relatively longer dsRNA, such as long synthetic poly I:C rather than ssRNA. In addition, Mda5 recognizes and defenses the infection of picornavirus; a non-enveloped positive sense single-stranded RNA virus, including Encephalomyocarditis virus (EMCV), Mango virus, and Thyler's virus (Kato et al. 2006; Loo et al. 2008).

4.2. MAVS/VISA/Cardif/IPS-1, the adaptor protein for RLR signaling pathway

MAVS (also termed VISA/Cardif/IPS-1), a mitochondrial outermenbrane protein, is an essential adaptor protein for the RLR-, TLR3-, and TLR4-mediated IFN-β promoter activation. In RNA virus-infected cells, MAVS, activated by the association of the RNA-bound RLRs through the CARD-CARD interaction, activates transcriptional factors, including both IRF3 and NF-κB that collaborate to induce IFN-β, by unknown mechanisms. The production of IFN-β mRNA is severely impaired in MVAS$^{-/-}$ cells either infected with various RNA viruses or treated with dsRNA or lipopolysaccharide (LPS) (Kumar et al. 2006; Sun et al. 2006).

The mitochondrial localization of MAVS is essential for the induction. The deletion of the C-terminal transmembrane (TM) domain in MAVS abrogates MAVS-dependent IFN-β induction (Seth et al. 2005). This release is mediated by the NS3-4A

protein of hepatitis C virus (HCV) (Li et al. 2005) resulting in the induction of IFN-β (Meylan et al. 2005; Loo et al. 2006). Cleavage-dependent abrogation of innate immunity is also observed in cells infected with other viruses (Chen et al. 2007; Yang et al. 2007). However, two recent studies suggest that the TM domain-mediated dimerization, rather than mitochondrial localization, of MAVS is required for the MAVS dependent signaling pathway. Thus a single amino acid mutant MAVS (Q512L), which is constitutively dimerized and delocalized from mitochondria, efficiently activates IFN-β promoter without virus infection (Baril et al. 2009). Artificially forced dimerization of MAVS is sufficient for its activation without mitochondrial localization (Tang and Wang 2009).

Recently, MITA/STING or TRAF3 has been reported as a connector between MAVS and TANK binding kinase 1 (TBK1), the downstream kinase of IRF3 (see below), in the signaling pathway. MITA/STING localizes to either mitochondrial outer membrane or to the endoplasmic reticulum (ER) membrane, associates with RIG-I and MAVS, and then recruits TBK1, thereby facilitating phosphorylation of IRF3 by TBK1 (Zhong et al. 2008). MITA/STING that is on the ER membrane forms a large complex (including other components) that also associates with, and activates TBK1 (Chien et al. 2006; Ishikawa and Barber 2008). MITA/STING$^{-/-}$ mice cannot efficiently induce IFN-β when infected by various pathogens, and as result develop much more serious disease (Ishikawa and Barber 2008).

TRAF3, an E3 ubiquitin ligase that conjugates K63-linked polyubiquitin chains, associates through its TRAF domain with the TRAF interacting motif (TIF) within MAVS (Saha et al. 2006). TRAF3 recruits TBK1 and activates IRF3, and TRAF3$^{-/-}$ cells

show severely impaired production of type I IFNs in response to virus infection (Oganesyan et al. 2006). The Gn protein cytoplasmic domain of NY-1 Hantavirus, a enveloped segmented negative sense RNA virus, blocks the induction of IFN-β by interfering with the association of TRAF3 and TBK1 (Alff et al. 2008).

4.3. TBK1, a kinase phosphorylating IRF3

TBK1 phosphorylates IRF3 or IRF7 to activate IFN-β gene transcription in virus-infected cells. TBK1, a noncanonical I-*κ*B kinase (IKK) which is expressed constitutively in all cell types, is activated by phosphorylation of its serine at position 172 by an unknown kinase *in vivo* (Kishore et al. 2002). Activated TBK1 phosphorylates a cluster of serine/threonine residues in the IRF3 protein at C-terminus (between residues 385 and 405). Experiments carried with $TBK1^{-/-}$ cells clearly show that TBK1 is the upstream kinase for the IRF3 signaling pathway (Hemmi et al. 2004; McWhirter et al. 2004).

4. 4. IRF3, a transcription factor, and enhanceosome

IRF3 is constitutively expressed in cytoplasm of various tissues and maintained in a latent conformation. The virus-induced phosphorylation of IRF3 by TBK1 is followed by its dimerization and nuclear accumulation. The activated IRF3 and NF-κB combine with AP-1, and CREB-binding protein (CBP) to form enhanceosome that binds to the IFN-β gene promoter and stimulates the transcription of IFN-β gene.

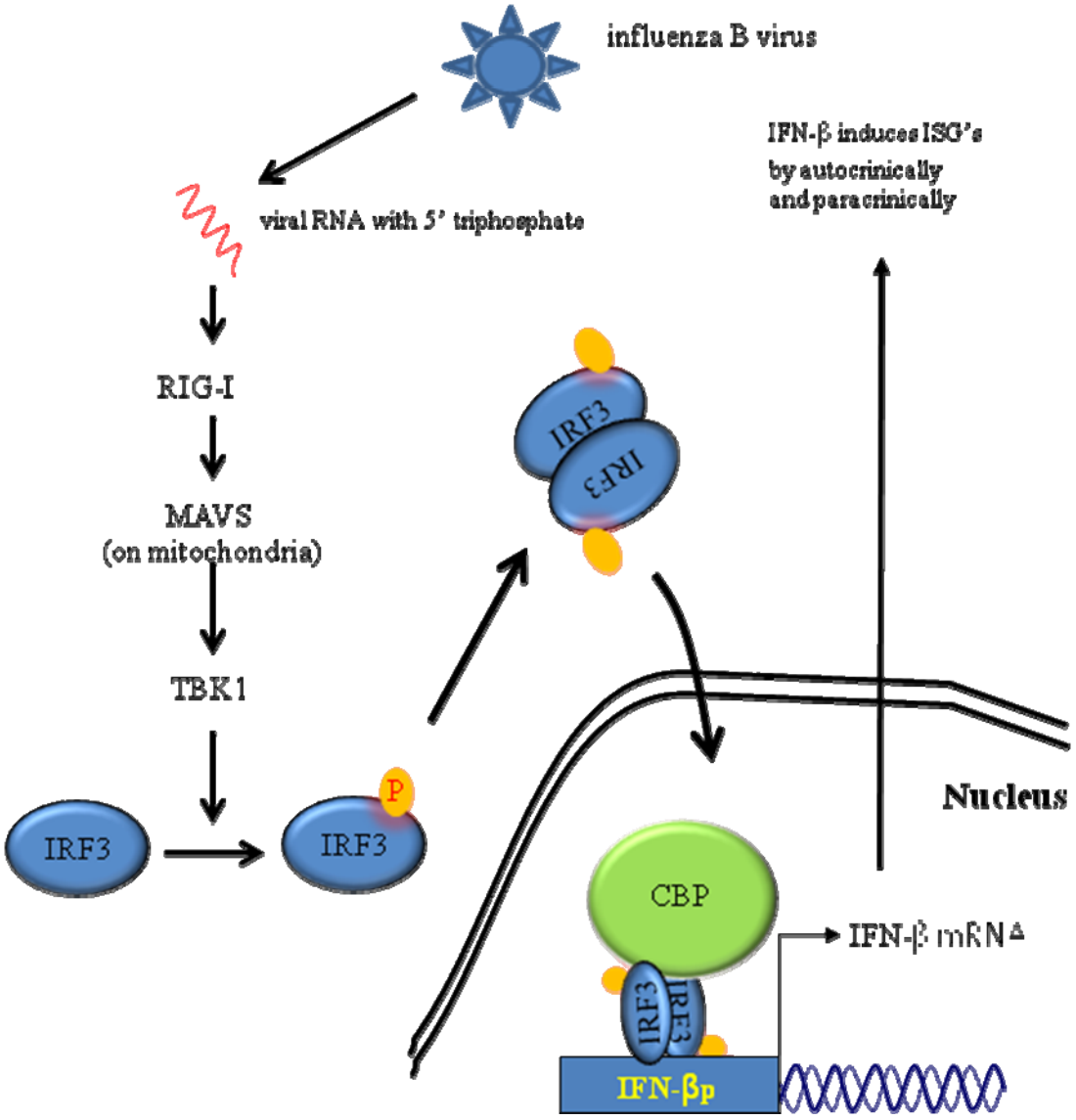

Figure 1.9 Production of IFN-β mRNA in cells infected with influenza B virus The engagement of vRNAs with 5' terminal triphosphate to the RIG-I protein induces a conformational change to expose its N-terminal CARD domains which associates with MAVS on mitochondria via the CARD-CARD interaction. The activated MAVS activates TBK1 to phosphorylate IRF3, the major transcription factor. The phosphorylated IRF3 are dimerized, accumulated in the nucleus and associated with CBP, the transcriptional co-activator. The complex of CBP-IRF3 dimer forms enhanceosome by combining the other transcription factors, NF-κB and AP-1, and turns on the transcription of the IFN-β genes.

4. 5. Regulation of the IFN-β induction pathway by cellular proteins (Figure 1.10)

Suppressor of IKK*i* (SIKE) interacts with TBK1 and IKK*i* constitutively but the association is disrupted in the cells infected with VSV. SIKE is contained in a complex containing TBK1 in resting cells; however it is dissociated from the complex in cells infected with VSV. When SIKE is overexpressed, SIKE specifically interferes with the RLR-dependent activation of IRF3 by virus infection. The knock down of endogenous SIKE potentiates cellular antiviral responses by enhancing the production of IFN-β in VSV-infected cells (Huang et al. 2005). Accordingly, SIKE may interfere with the activation of TBK1 in resting cells by sequestering it into an inactive complex but more studies are required to elucidate the mechanism.

A third member of the inducible RLR family, LGP2, which lacks N-terminal CARD domains, negatively regulates virus-dependent IFN-β induction. According to early studies, LGP2 sequesters viral RNAs away from RIG-I or Mda5 (Rothenfusser et al. 2005; Yoneyama et al. 2005). As another inhibitory mechanism, LGP2 interferes with the multimerization of RIG-I, dubbed as RIG-I active form, by interacting RIG-I via LGP2 C-terminal region, comparable to the RIG-I RD (Saito et al. 2007).

The tripartite motif protein 25 (TRIM25) positively regulates the virus-dependent IFN-β induction. TRIM25 catalyzes K63-linked ubiquitination of the lysine residue at 172 (K172) amino acid, which is in the N-terminal CARD domain of RIG-I. The ubiquitinated RIG-I binds MAVS more efficiently, thereby increasing the production of IFN-β (Gack et al. 2007).

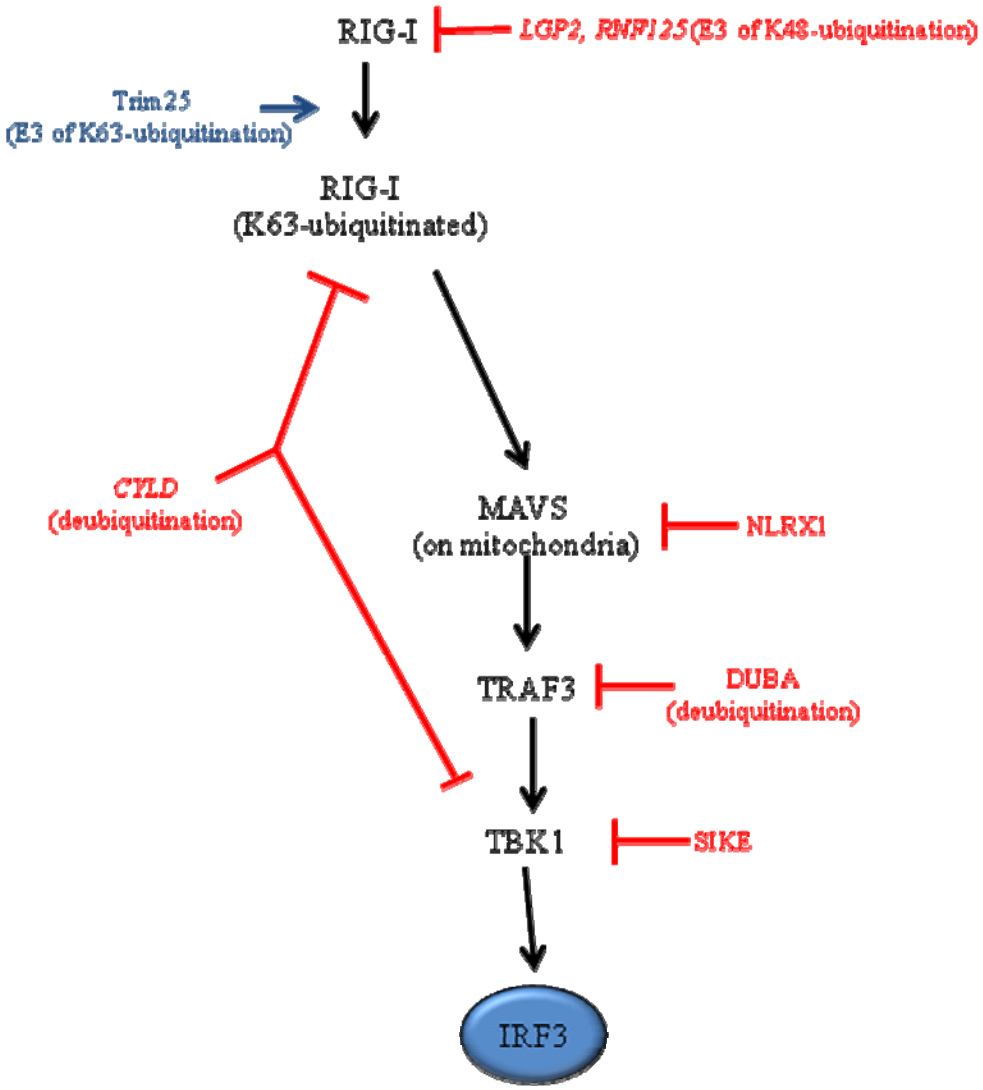

Figure 1.10 Cellular factors which regulate RLR pathway The RLR pathway is tightly regulated by many cellular factors. Some factors are constitutively expressed but some factors (written in italic type) are inducible and they affect on the pathway as negative feedback regulators. Some factors (written in blue) regulate positively but some (written in red) regulate negatively.

Deubiquitinating enzymes (DUB), CYLD and DUBA, negatively regulate virus-dependent IFN-β induction by removing K63-linked conjugated ubiquitin from target molecules (Kayagaki et al. 2007; Friedman et al. 2008).

A RING finger family protein, RNF125 is another regulator of virus-dependent IFN-β induction. RNF125 is an IFN inducible protein and an E3 ubiquitin ligase that directs proteasomal degradation of target proteins. RNF125 interacts with and ubiquitinates RIG-I, resulting in the degradation of RIG-I. In addition, RNF125 ubiquitinates other proteins in the signaling pathway, Mda5 and MAVS (Arimoto et al. 2007). RNF125 targets multiple proteins in the IFN-β induction pathway by regulating their abundance. Like LGP2, RNF125 might function as a negative feedback regulator in the RIG-I dependent signaling pathway because it is induced by IFN-β.

NLRX1 is a member of the highly conserved NLR family (nucleotide-binding domain (NBD) - and leucine-rich-repeat (LRR) -containing family) and a mitochondria-localized protein. The NLRX1, constitutively expressed, interacts with MAVS on mitochondrial outer membrane in uninfected cells. Overexpression of NRLX1 disrupts the virus-induced interaction between MAVS and RIG-I and concomitantly blocks the virus-induced IRF3 dimerization. In contrast, the depletion of endogenous NRLX1 using siRNA potentiates the virus-mediated IFN-β production and antiviral innate immunity (Moore et al. 2008). Taken together, the data indicate that NRLX1 might sequester MAVS away from RIG-I thereby preventing the RIG-I signaling but more studies are required to understand why this negative regulation is present.

5. Various mechanisms avoiding IFN-β induction by viruses (Figure 1.11)

Mammals have been threatened by various pathogenic viruses and have evolved antiviral innate immunity to combat these pathogens. Viruses are obligate parasites that require the host system for survival and have evolved various strategies to overcome the innate immunity to efficiently replicate themselves. The study of viral evasion mechanisms can provide us with clues to develop antiviral drugs that block viral evasion mechanisms.

5.1. Modification or sequestration of viral RNAs

Several viruses modify 5' triphosphate of viral RNAs to evade the surveillance of RIG-I. Some viruses trim the 5' nucleotide(s) of viral RNA with an endonuclease activity encoded by them (Habjan et al. 2008). As another mechanism, many viruses encode dsRNA binding proteins which can sequester dsRNA from the detection of RLRs. Vaccinia virus E3L and Ebola virus VP35 are good examples (Xiang et al. 2002; Cardenas et al. 2006)

5.2. Interference of the component of the RLR signaling pathway

RLRs

RLRs are targeted and the RLR dependent signaling is blocked by viral proteins. The NS2 protein of respiratory syncytial virus (RSV), a negative-sense, single-stranded RNA virus of the family *Paramyxoviridae*, interferes with the association of RIG-I and MAVS by binding the CARD domain of RIG-I (Ling et al. 2009). The V proteins of many paramyxoviruses specifically interfere with the Mda5 signaling pathway by binding the helicase domain of Mda5, and inhibiting Mda5 self-association (Childs et al. 2009). Poliovirus and EMCV, positive single-stranded RNA viruses, induce the degradation of Mda5 mediated by proteasome and caspase in the virus-infected cells (Barral et al. 2007). However, the instability might be an accompanying effect of poliovirus-dependent apoptosis because any responsible viral proteinases have not been identified and the treatment of caspase inhibitor decreases the degradation. Recently, Trim25, a positive regulator of virus-mediated IFN-β induction by ubiquitinating RIG-I, has been reported to be targeted by the NS1 protein of the laboratory-generated influenza A/PR/8/34 strain. The interaction of the NS1A protein and Trim25 inhibits the Trim25-mediated ubiquitination of the RIG-I CARD domain and the subsequent IRF3 activation. The binding of TRIM25 requires the dsRNA-binding activity of the NS1 protein, indicating that it is at least in part mediated by dsRNA rather than by a direct protein-protein interaction. Replacement of the two NS1 E residues at positions 96 and 97 also eliminate TRIM 25 binding. This loss of binding may be due at least in part to disruption of the secondary structure of the effector domain, because the E97A substitution eliminates a crucial salt bridge in the effector domain. More IFN-β proteins are produced in cells

infected with a recombinant influenza A/PR/8/34 virus with the E96A, E97A double mutation (Gack et al. 2009).

MAVS

MAVS is targeted by many viral proteases including hepatitis A virus (HAV), a non enveloped single-stranded RNA virus of *Picornaviridae*, 3ABC protein (Yang et al. 2007) and NS3-4A protein of HCV (Meylan et al. 2005), a small enveloped positive sense single-stranded RNA of family *Flaviviridae*, and GB virus B, a relative of HCV (Chen et al. 2007). In addition, NS3-4A of HCV targets TRIF, the essential adaptor protein in the TLR3 signaling pathway (Li et al. 2005).

TBK1

TBK1 is targeted by several viral proteins. The P protein of borna disease virus (BDV), a nonsegmented negative sense single-stranded RNA virus, targets the kinase activity of TBK1 by interaction thereby interfering with the activation of IRF3 (Unterstab et al. 2005). The P protein of rabies virus, a negative sense single-stranded RNA virus of family *Rhadoviridae*, blocks IFN-β induction by blocking the TBK1-mediated phosphorylation and the subsequent dimerization of IRF3 (Brzozka et al. 2005). Additionally, the P protein interacts with STAT1 and prevents IFN-dependent activated STAT1 from localizing to the nucleus (Vidy et al. 2005).

A synergistic factor of the TBK1 activity can be targeted by a viral protein. The K7 protein of Vaccinia virus (VACV), an enveloped virus with double-stranded DNA as

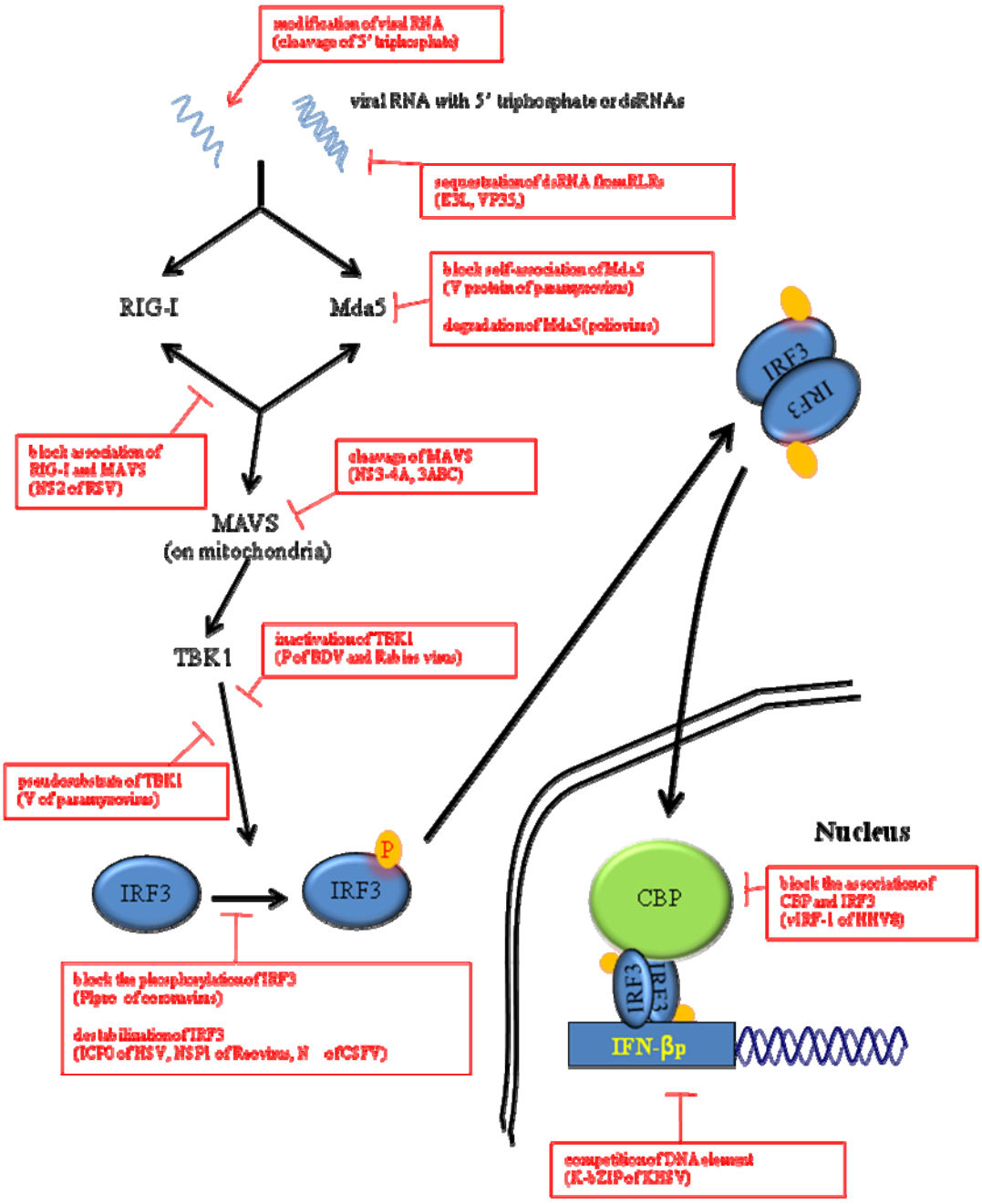

Figure 1.11 Inhibitory mechanisms of RLR pathway by viral proteins in virus-infected cells In virus-infected cells, viral proteins block innate immunity by blocking the induction of the IFN-β gene via targeting various steps in the pathway. A viral protein can target multiple steps in the pathway (V protein of paramyxovirus).

genome, interferes with the activation of IRF3 in virus-infected cells. Instead of TBK1, the K7 protein targets DDX3, a cellular DEAD box helicase, which synergistically enhances the virus-dependent or the TBK1-dependent activation of IFN-β promoter by an unknown mechanism. DDX3 transiently interacts with IKK*i* in cells infected with viruses (Schroder et al. 2008). The K7 protein may compete with TBK1 for a binding site in DDX3 required for the synergistic effect in virus-infected cells. However, the mechanism of the synergistic activation of IFN-β promoter by DDX3 requires more studies.

IRF3

IRF3 is targeted through various mechanisms. Some viral proteins block the phosphorylation of IRF3 by functioning as a pseudosubstrate of TBK1 or IKKi. V proteins of paramyxoviruses including mumps virus (MuV), human parainfluenza virus 2 (hPIV2), and parainfluenza virus 5 (PIV5) are examples. The V proteins of those viruses are directly phosphorylated by TBK1 and IKKi. The phosphorylated V proteins are less stable and are degraded (Lu et al. 2008). In addition, the V proteins target the activation of STAT with various mechanisms including the sequestration of the phosphorylated STAT from nuclei and the ubiquitination-dependent degradation of STAT by recruiting ubiquitin E3 ligase complex consisting of DDB1-Cul4A (Horvath 2004).

The phosphorylation of IRF3 is also blocked by viral proteins directly bind to it. The papain-like protease domain (PLpro) of severe acute respiratory syndrome coronavirus (SARS-CoV), an enveloped positive sense single-stranded RNA virus of

family *Coronaviridae*, interacts with IRF3 and ablates IRF3-phosphorylation (Devaraj et al. 2007).

Other viral proteins dynamically regulate the stability of IRF3 through interaction. The ICP0 protein of Bovine Herpesvirus 1 (bICP0) induces the degradation of IRF3 but not IRF7 (Saira et al. 2007). NSP1, a nonstructural protein, of rotavirus, a segmented double stranded RNA virus in the family *Reoviridae*, binds IRF3 and causes the proteasome-mediated degradation of IRF3 (Barro and Patton 2005). The N^{pro} protein of classical swine fever virus (CSFV) causes the proteasomal degradation of IRF3 in infected cells through interaction (Bauhofer et al. 2007).

Some viral proteins ablate a downstream event after activation of IRF3. The vIRF-1 protein encoded by human Herpes Simplex Virus 8 (HHV8) abrogates the association of the activated IRF3 and the CBP/p300 by binding CBP/p300 and thereby interferes with the formation of the enhanceosome (Lin et al. 2001). Similarly, the vIRF3 protein encoded by Kaposi's sarcoma associated herpes virus (KSHV) interacts with the DNA-binding domain of IRF7 and inhibits its DNA binding activity (Joo et al. 2007). K-bZIP, a viral transcription factor, encoded by KSHV, competes for same the DNA binding site in the IFN-β promoter with the activated IRF3, and as a result blocks the activation of the IFN-β promoter by preventing IRF3 from binding to the promoter (Lefort et al. 2007).

The NS1A proteins of almost all human influenza A viruses bind 30kDa components of CPSF complexes thereby suppressing the production of cellular proteins including IFN-β (Das et al. 2008) in infected cells by inhibiting a general

posttranscriptional step, the 3'-end processing of cellular pre-mRNAs. Therefore, generally, IRF3s are activated in cells infected with human influenza A viruses. However, uniquely, influenza A/PR/8/34 virus, of which the NS1A protein cannot interact with CPSF complexes in infected cells, suppresses the production of IFN-β mRNA via inhibition of IRF3 activation in the virus infected cells (Das et al. 2008). However, the suppressing mechanism or a responsible viral protein has not yet been determined.

CHAPTER TWO

IDENFIFICATION OF BRD2 AS A CELLULAR PROTEIN THAT INTERACTS WITH THE EFFCTOR DOMAIN OF INFLUENZA B VIRUS NS 1 PROTEIN (NS1B PROTEIN)

INTRODUCTION

Influenza B virus is one of influenza viruses, classified as type A, B, and C, based on antigenic differences of NP and M proteins. Influenza B viruses are a member of the family *Orthomyxiviridae*, has 8 segmented, negative sense, single-stranded RNA genomes. Influenza B viruses cause a highly contagious respiratory disease in humans and account for 30 ~ 40% of the seasonal infections caused by influenza virus in the United States during last 9 seasons (From CDC website). Influenza B viruses, whose host range is almost limited to humans, have never caused pandemics.

The nonstructural protein 1 of influenza B virus (the NS1B protein) is encoded by the smallest gene segment which also encodes the NS2B protein by alternative splicing mechanism (Racaniello and Palese 1979). The NS1B protein is a multifunctional protein which interacts with RNA or cellular proteins and is expressed in infected cells, but is not incorporated into virions. The RNA binding domain (RBD) comprising the N-terminal 93 amino acids of NS1B is functionally and structurally similar to that of NS1A

(Chien et al. 1997; Liu et al. 1997; Yin et al. 2007). They fold a unique six-helical homodimer structure. The dsRNA-binding surface comprises conserved hydrophilic and hydrophobic residues (Yin et al. 2007). The arginines at amino acids 50 and 53 of NS1B are essential for NS1B RBD-dsRNA interaction, and the arginine at amino acid 38 and the lysine at amino acid 41 of NS1A are essential. The RBD of both proteins primarily target A-form dsRNA without sequence specificity (Wang and Krug 1996).

In contrast, the function of the NS1B effector domain is completely different from that of NS1A. More functions of the NS1A effector domain have been revealed. The effector domain of NS1A binds several cellular proteins including: the cellular 30-kDa component of the cleavage and polyadenylation specificity factor (CPSF30), a required cellular factor for the 3′-end processing of cellular pre-mRNAs, thereby inhibiting the production of all cellular mRNAs, including interferon-β (IFN-β) mRNA (Nemeroff et al. 1998; Das et al. 2008); the 85 kDa regulatory subunit (p85β) of phosphatidylinositol-3-kinase (PI3K), resulting in the activation of PI3K/Akt pathway thereby blocking premature induction of apoptosis in the influenza A virus-infected cells (Hale et al. 2006; Ehrhardt et al. 2007b); and PKR, resulting in the inhibition of PKR activation which blocks all protein synthesis via PKR-mediated phosphorylation of eIF2α (Li et al. 2006; Min et al. 2007). The functions described above are not conserved in the NS1B effector domain.

A major function of the effector domain of the NS1B protein is to inhibit the production of IFN-β mRNA. A recombinant influenza B virus encoding only the N-terminal 104 (104 virus) or 110 amino acids of the NS1B protein is attenuated 10 to 100

folds coupled with increased production of IFN-β mRNA (Dauber et al. 2006; Hai et al. 2008). However, the mechanism(s) by which the effector domain of the NS1B protein inhibits the production of IFN-β mRNA has not been established.

The NS1B protein blocks ISG15 conjugation in virus-infected cultured human cells, as another function, although the function is not exclusively mediated by the NS1B effector domain. ISG15, the IFN-inducible, ubiquitin-like 15kDa protein, is the only identified NS1B-interacting cellular protein and ISG15 binding to the NS1B protein requires the N-terminal 103 amino acids of NS1B (Yuan and Krug 2001). ISG15 is conjugated to at least 158 target molecules by E1 (Ube1L), E2 (UbcH8), and E3 (Herc5) enzymatic cascade (Yuan and Krug 2001; Zhao et al. 2004; Zhao et al. 2005; Wong et al. 2006). Interestingly, the NS1B protein interacts with human ISG15 but not with mouse ISG15 (Sridharan, P., Zhao, C. and Krug, R, M., unpublished experiments), therefore, influenza B virus is susceptible in wild type mice and less susceptible in $ISG15^{-/-}$ or $Ube1L^{-/-}$ mice like several viruses (Lenschow et al. 2007; Lai et al. 2009).

IFN-β establishes innate immunity against viral pathogens by inducing several IFN stimulated genes (ISGs) which interfere with the viral replication in IFN-β treated cells (Borden et al. 2007; Sadler and Williams 2008). Many viruses have evolved various mechanisms by which they inhibit the production of IFN-β (Chapter one, Figure 1.11). The NS1 proteins of influenza viruses use two mechanisms to suppress the production of IFN-β proteins. First, 3'-end processing of IFN-β pre-mRNA is targeted by NS1 proteins as a part of the viral inhibitory mechanism to affect on that of total cellular pre-mRNA. Most human influenza A viruses employ this mechanism (Das et al. 2008). The other

mechanism is to suppress the production of IFN-β mRNA by inhibiting the IRF3 activation, a major transcription factor for the induction of INF-β in virus infected cells. The NS1 proteins of influenza B viruses and influenza A/PR/8/34 viruses use this mechanism.

Clearly, the effector domain of NS1B takes charge in the suppressed production of IFN-β mRNA in virus-infected cells based on previous experiments using recombinant influenza B viruses encoding a truncated (1-104-amino acid-long) NS1B protein (Dauber et al. 2006; Hai et al. 2008). However, the studies do not reveal any mechanism. Furthermore, the known NS1B functions are not correlated with the suppression. The low level activation of IFN-β promoter and the directly correlated less IFN-β expression in cells infected with recombinant influenza B viruses encoding several types of mutant NS1B proteins lacking dsRNA binding activity in the mutant virus-infected cells demonstrate dsRNA binding is not essential for the suppression of the production of IFN-β mRNA in cells infected with influenza B virus (Dauber et al. 2006). More IRF3 dimers and IFN-β mRNA are detected in cells infected with viruses whose NS1B (1-104) protein can interact with ISG15 indicating that ISG15 binding does not play a role in suppressing IFN synthesis (Chen Zhao's unpublished data, Chapter III Figure 3.10). Moreover, in our lab, the essential NS1B amino acids for ISG15 binding have been determined and a recombinant influenza B virus encoding the mutant NS1B protein suppressed the activation of IRF3 in infected cells like wild type influenza B virus (Chen Zhao's unpublished data). The data indicate that the activity of ISG15 binding to NS1B does not mediate the suppression. Taken together, data implicate a domain within the region

spanning amino acids 105 to 281 of NS1B mediates the suppression of the production of IFN-β mRNA in infected cells.

We hypothesized the NS1B protein functions by binding specific cellular proteins as is the case for the NS1A protein. To elucidate the mechanism by which the NS1B effector domain carries out its functions, we screened for cellular interacting proteins using affinity purification, followed by mass spectrometry. This approach led to the identification of Brd2, which specifically interacting with a region of the effector domain of the NS1B protein, *in vivo*, *in vitro*, and in virus-infected cells.

MATERIALS AND METHODS

Cell lines A549 (human lung carcinoma cells), MDCK (Madin-Darby canine kidney cells), COS7 (African Green Monkey SV40-transfromed kidney fibroblast cells), HEK293T (human embryonic kidney cells), and HeLa (human cervical cancer cells) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics at 37°C under a 5% CO_2 atmosphere.

Generation of recombinant influenza B/Yamanashi/166/98 virus by transfecting eight plasmids A wild type (wt) recombinant influenza B virus was generated by transfecting eight plasmids containing the full-length cDNAs for each of eight influenza B/Yamanashi/166/98 viral genomic sequences into the transiently co-cultured COS7 cells

and MDCK cells and at various time post transfection, culture supernatants were collected as previously described (Hoffmann et al. 2002). To generate a recombinant virus encoding an N-terminal double polyoma tagged NS1B protein (MEYMPME, Py tag) (PyBYNS virus), the plasmid encoding wt NS gene was replaced with the plasmid containing a modified NS gene by adding the sequence of the Py tag to the DNA sequences between 5' untranslational region (5' UTR) and the start codon for the NS1B protein. The insertion was performed by using two rounds of PCR and two long forward primers: 5'-AGA AAA AAT GGA ATA TAT GCC AAT GGA AAT GGA ATA TAT GCC AAT GGA AAT GGC GGA CAA CAT GAC CAC-3' and 5'-CGT CTC AGG GAG CAG AAG CAG AGG ATT TGT TTA GTC ACT GGC AAA CAG AAA AAA TGG AAT ATA TGC-3'. The generated DNA was sequenced and cloned into pAD3000 plasmid. The generated viruses were tittered by plaque assay on MDCK cells and individual plaques were amplified in 10-day-old embryonated chicken eggs at 34°C for 2 days. The amplified viruses were tittered by plaque assay. Viral RNA segments were sequenced.

Plaque assay Confluent monolayer MDCK cells were incubated with 10 fold serially diluted viruses in serum-free DMEM at 34°C under a 5% CO_2 atmosphere for 1 hour to adsorb viruses. The inoculums were removed and the cells washed with sera free DMEM twice. The washed cells were covered with DMEM containing, 1% agarose, antibiotics, and 1 μg/ml N-acetyl trypsin (NAT). The covered cells were incubated at 34°C under a 5% CO_2 atmosphere. The covered agarose was removed and cells were stained with

staining solution containing 0.1% Naphthalene Blue Black, 0.5M sodium acetate, 6% glacial acetic acid (v/v) and the number of plaques were counted to determine titers.

Multiple cycle virus infection Confluent monolayer MDCK cells were infected with viruses at a multiplicity of infection (moi) of 0.001 as described in the plaque assay protocol. The infected cells were incubated in 5ml DMEM media supplemented with 1 μg/ml NAT at 34°C under a 5% CO_2 atmosphere. Viruses in the culture supernatants, collected every 12 hours, were tittered by plaque assay.

Affinity purification of NS1B interacting proteins in A549 cells infected with recombinant influenza B viruses 2×10^7 confluent monolayer A549 cells were infected with wt influenza B viruses or PyBYNS viruses at a moi of 10 and lysed in 1ml 1% NP40 lysis buffer (50mM Tris·HCl (pH8.0), 150mM NaCl, 1mM EDTA, 1% NP40, 1mM PMSF, 1×complete protease inhibitor cocktail (Roche)) at 10 hours post infection. The lysates were incubated with 50μl (bed volume) anti polyoma affinity resin prepared by cross-linking anti polyoma antibodies to protein G Sepharose resins (Amersham) as previously described (Stevens 2000) on rotation platform overnight at 4°C. The incubated resins were washed 3 times with 1ml lysis buffer and once with 1ml D100 buffer (20mM HEPES (pH7.9), 100mM NaCl, 8% glycerol, 10mM β-Mercaptoethanol, 1mM PMSF, and 1 ×compete protease inhibitor cocktail). Proteins on the resins were eluted with 50μl D100 buffer containing polyoma peptide (0.5μg/ml) (Invitrogen) overnight at 4°C, resolved by SDS-PAGE, stained with 0.1% coomasie blue in solution (50% methanol,

10% glacial acetic acid) for 1 hour at room temperature, and destained with wash solution (50% methanol, 10% glacial acetic acid). The specific bands that only appear in the lane on which we loaded the purified elutes from the A549 cells infected with PyBYNS viruses were cut and sent the Keck facility in the Yale University for mass spectrometry.

Double affinity purification of NS1B-interacting proteins in 293T cells transfected with plasmids encoding the NS1B protein with double affinity tags Subconfluent 293T cells on 10cm plate were transiently transfected with 10μg plasmids expressing only double tag, the full length NS1A proteins or the NS1B proteins containing two affinity tags, N-terminal GST tag and C-terminal Flag: the full-length NS1B protein, the effector domain (ED) or RNA binding domain (RBD) of the NS1B protein under the control of constitutively active CMV promoter (Figure 2.1) and lysed in 1ml NP40 lysis buffer (50mM Tris·HCl (pH7.5), 150mM NaCl, 5mM EDTA, 2.5mM $MgCl_2$, 1% NP40, 10% glycerol, 1mM PMSF, 1×complete protease cocktail) at 48 hours post transfection. Each lysate was diluted with 3ml 0.5% NP40 wash buffer (10mM Tris·HCl (pH 7.5), 0.5% NP40, 150mM NaCl) and subjected to glutathione affinity chromatography. Proteins on the resins were eluted with 200μl GST elution buffer (50mM Tris·HCl (pH 8.0), 150mM NaCl, 0.5% Triton X-100, 10% glycerol, 1×complete protease inhibitor cocktail) containing 10mM glutathione (Sigma) twice. The eluents were incubated with 25μl anti Flag M2 agarose resin (bed volume) (Sigma) overnight in cold room with rotating. The incubated resins were washed with 1ml 0.5% NP40 wash buffer 3 times and 1ml GST elution buffer twice. Proteins on the anti Flag M2 affinity resins were eluted

with 25μl Flag elution buffer (50mM Tris·HCl (pH7.5), 150mM NaCl, 0.012% Triton X-100, 10% glycerol, 0.2mM EDTA) containing 1mg/ml 3XFlag peptide (Sigma) twice, and were resolved by SDS-PAGE. The gel was stained using Silver Quest silver staining kit (Invitrogen). For mass spectrometry, the scale up purification was carried out. 293T cell lysates were prepared from the pooled transfected cells, grown on five 10cm plate. Proteins in the final eluents were concentrated with Centricon 25 column (Amicon) and subjected to SDS-PAGE. The gel was stained using colloidal blue staining kit (Invitrogen). Specific bands were cut and sent to the Taplin Mass Spectrometry Facility in the Harvard University for mass spectrometry.

***In vitro* GST pull down assay** To prepare GST fusion NS1 proteins, genes of the NS1A protein and the wild type, the RNA-binding-defective, or the NS1B effector domain were cloned into pGEX3X plasmid. GST fusion proteins were expressed and purified as previously described (Qiu and Krug 1994). To prepare the labeled proteins, pcDNA3-mycBrd2 plasmid encoding the myc tagged Brd2 protein (kindly gifted from Dr. T. Schulz, Institute Hannover Medical School) was *in vitro* transcribed and translated using TNT Coupled Transcription/Translation kit (Promega) in the presence of [^{35}S] promix (Amersham). 2μg prepared GST fusion NS1 proteins were combined with the ^{35}S-labeled myc tagged Brd2 protein and the mixture was subjected to glutathione sepharose affinity selection as previously described (Nemeroff et al. 1995).

Determination of a cellular interaction using GST pull down assay Subconfluent 293T cells on 6-well plate were co-transfected with 1μg pcDNA3-mycBrd2 and 1μg one of plasmids encoding GST or several N-terminal GST fusion NS1 proteins as shown in Figure 2.1. Extracts from the transfected cells were prepared with 300μl NP40 lysis buffer and incubated with 25μl (bed volume) glutathione sepharose resin (Amersham) on rotation platform overnight 4°C after ¼ dilutions with 0.5% NP40 wash buffer. Bound proteins were subjected to SDS-PAGE after the resin washed extensively with 1ml 0.5% NP40 wash buffer 4 times and with 1ml GST elution buffer twice. The Brd2 protein, co-purified by GST fusion NS1 proteins, was detected by Western blotting.

Co-immunoprecipitation assay For co-immunoprecipitation assay, the Flag tag was fused to the Brd2 protein at N-terminus by inserting the Flag tag containing DNA fragment into pCN-mycBrd2 plasmid. HeLa cells, grown on 6cm plate to 80% confluence, were transfected with 3μg pCN-FlagBrd2, followed by the infection of the indicated influenza B viruses as a moi of 10 at 24 hours post transfection. Extracts were prepared with 500μl NP40 lysis buffer at 10 hours post infection and incubated with 30μl anti Flag M2 agarose resin (bed volume) overnight in cold room after diluting the lysate with 500μl 0.5% NP40 wash buffer. Proteins on the resins were washed as described *in vivo* GST pull down assay and were eluted with 30μl Flag elution buffer containing 1mg/ml 3×Flag peptide. The co-immunoprecipitated NS1B proteins were detected by Western blotting using polyclonal anti NS1B antibody.

Western Blotting Proteins, co-purified or directly extracted from cells, were resolved by SDS-PAGE and electrophoretically transferred on nitrocellulose membrane. Blots were immunostained with anti NS1B antibody, anti Flag antibody (Sigma), anti myc antibody (Roche), or anti GST antibody (company) as needed, and visualized by autoradiography after treatment ECL solution (Amersham).

RESULTS

Attempt to use a virus expressing an N-terminal tagged NS1B protein to identify cellular proteins that interact with the NS1B protein

To screen for cellular proteins that interact with the NS1B protein during influenza B virus infection, a recombinant influenza B/Yamanashi/166/98 virus expressing the N-terminal double polyoma (Py tag) tagged NS1B protein (PyBYNS virus) was generated by using eight-plasmid transfection method as previously described (Hoffmann et al. 2002). To determine whether PyBYNS virus is attenuated, we compared the difference in growth kinetics between PyBYNS virus and wt virus in MDCK cells. Viruses in the collected culture supernatants were tittered after multiple cycle infection in MDCK cells. Almost same titer of PyBYNS viruses as that of wt viruses at plateau indicates that PyBYNS virus is not attenuated.

The purification approach using the recombinant virus has a few problems. First, NS2B is always co-purified. NS2B shares N-terminal 11 amino acids with NS1B because it is encoded from alternative spliced mRNA transcribed from a viral NS gene (Briedis

and Lamb 1982). Therefore, both NS1B and NS2B, encoded by the recombinant virus, have same N-terminal tag. Furthermore, NS2B interacts with viral proteins. NS2B can interact with M1 and viral RNPs including NP (Imai et al. 2003). NP proteins can interact with many cellular proteins (Portela and Digard 2002) as well as the viral RNA polymerase (Biswas et al. 1998; Newcomb et al. 2009). NS2B also interacts with cellular proteins, export mediating proteins (Paragas et al. 2001)**.** Therefore, there could be inevitable noise that did not allow us to identify cellular proteins that bind to NS1B in final eluents. In order to eliminate co-purified NS2B and its associated proteins, we attempted to generate a recombinant virus that encodes N-terminal tagged NS1B and C-terminal tagged NS2B by adding two different tags to each terminus of the viral NS gene. Technically, we cannot generate such virus.

Transfection of plasmids expressing the NS1B effector domain containing two affinity tags led to the identification of Brd2 as a cellular protein that interacts with the effector domain

We transfected 293T cells with pcDNA3-based plasmids expressing NS1B proteins containing two affinity tags, N-terminal GST tag and C-terminal Flag: the full-length NS1B protein or the effector domain (ED) of the NS1B protein (Figure 2.1). 293T cells were chosen because of its high transfectability and strong expression of a gene encoded by the plasmids. To eliminate the production of spliced NS2 mRNA when the full-length NS1B protein is expressed, we mutated the 3' splice acceptor site without changing the amino acid sequence of the NS1B protein. As a control, a plasmid

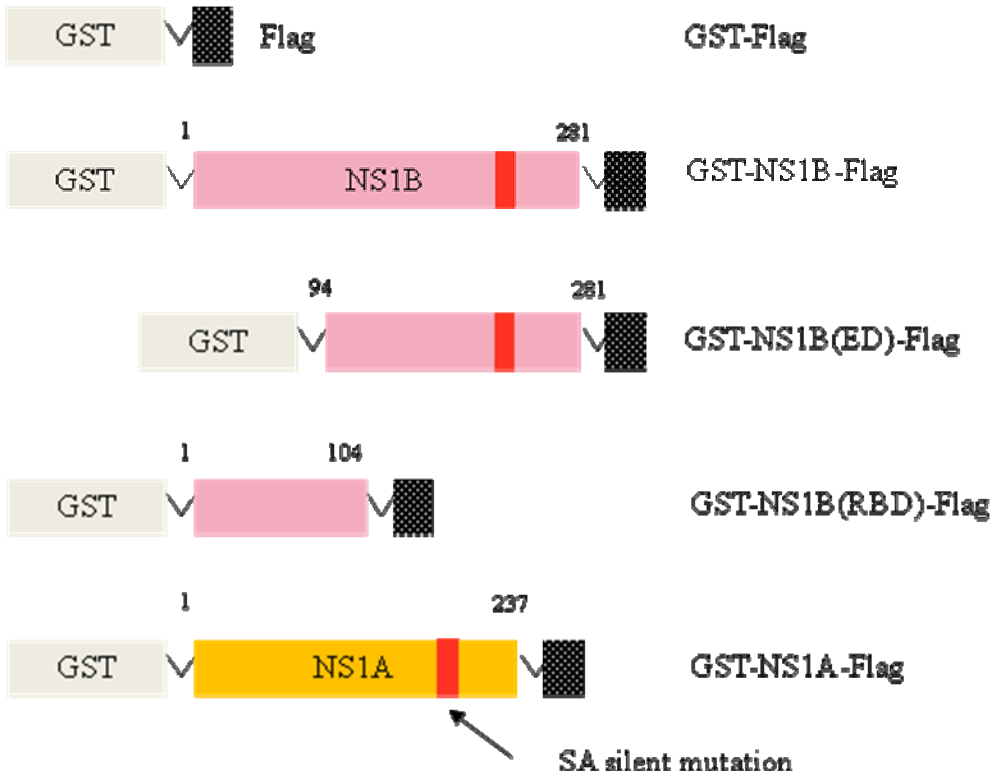

Figure 2.1 Schematic diagram of constructs A GST tag is added to N-termini and a Flag tag is added to C-termini for double affinity purification approach. To remove interference of encoded NS2B, we added silent mutation to the splicing accepter site (red rectangle) and it is designated as SA silent mutation. The NS1B genes are pink rectangle and NS1A gene is orange rectangle. The numbers above rectangles are the denoted amino acids.

expressing only GST-Flag fusion protein (GST- Flag) was used in this purification. To identify nonspecific RNA-mediated interacting factors, we transfected plasmids expressing the full length NS1A protein (GST-NS1A-Flag) and the RBD of the NS1B protein (GST-NS1B (RBD)-Flag). The double-tagged proteins and their associated proteins were purified from cell extracts by sequential round of affinity chromatography on glutathione sepharose and anti-Flag M2 agarose resins. After double affinity purification, three bands with molecular weight of 100, 115, and 160kDa were found only in the extracts from cells transfected the plasmids encoding the full length or the effector domain of the NS1B protein (marked with the red arrow head on lane 5 in Figure 2.2). The bands were excised and sent to the Taplin facility at the Harvard University for mass spectrometry analysis. Several proteins were identified in each band (Table 2.1).

Indeed, Nucleolin and DDX21 among identified proteins with more than 10 matched peptide sequences in their protein sequences were identified in previous approach using a virus encoding N-terminal tagged NS1B protein. As a result of further analyses, we found that they bind to NS1A as well as NS1B and RNA strongly mediates the interaction. The NOL1, a nucleolar protein like nucleolin and DDX21, is determined as an rRNA binding protein (Gustafson et al. 1998). The pinin protein has not been reported as an RNA binding protein but it forms complex with several RNA binding proteins (Wang et al. 2002; Sakashita et al. 2004), suggesting that the interactions with NS1B might be RNA-mediated.

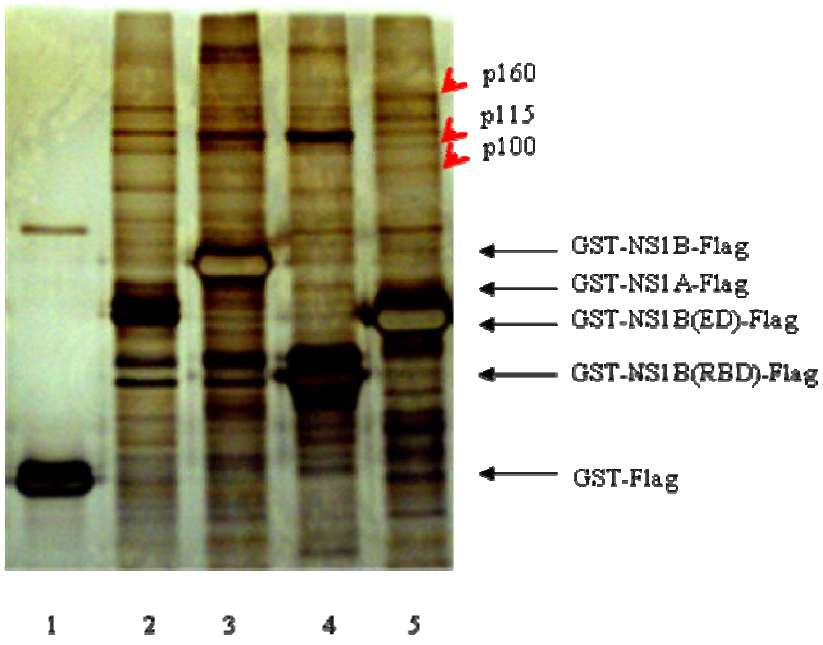

Figure 2.2 Three protein bands which specifically interact with the NS1B protein and the NS1B effector domain were detected. Proteins of the double affinity purification elute from 293T cells, transfected with the plasmids encoding GST-Flag protein (lane 1), double tagged NS1A protein (lane 2), or double tagged NS1B proteins, containing the full-length (lane 3), RBD (lane 4) or the effector domain (lane 5), were resolved on an 8% SDS-polyacrylamide gel and silver stained. Three indicated protein bands (with the red arrow heads) that exceptionally appear in both lanes 3 and 5 on which we loaded final eluents from cell extracts containing the double tagged NS1B proteins or the NS1B effector domains were cut and sent for mass spectrometry after a scale-up purification.

Among these, Brd2 was studied further. 13 peptide sequences are matched in the Brd2 protein sequence and covered 23% of the total protein sequences. Functionally, Brd2 is identified as a mitogen-activated nuclear serine/threonine kinase (Denis and Green 1996) possessing chromatin binding activity with a preference for acetylated lysine 12 on histone H4 (Kanno et al. 2004) and transcriptional activity that regulates the expression of cell cycle regulating proteins such as cyclin A, E, D, and DHFR via its association with transcriptional regulators such as E2F1 or 2 (Denis et al. 2000). Brd2 localizes to both the nucleus and cytoplasm in resting cells but mitogen treatment induces nuclear localization (Guo et al. 2000). Nuclear Brd2 facilitates the transcription of cyclin genes. However, the function of cytoplasmic Brd2 in resting cells has not been studied.

We chose Brd2 as a candidate because we hypothesized that the NS1B protein could interfere with Brd2-mediated cellular gene transcription and the inhibition of the IFN-β mRNA production could be a part of a NS1B-mediated inhibition of Brd2-dependent transcriptions. Furthermore, Brd2 has not been reported a RNA binding protein or an interaction with a RNA binding protein among identified proteins with more than 10 matched peptide sequences in their protein sequences (Table 2.1).

Brd2 specifically interacts with the effector domain of the NS1B protein *in vivo* and *in vitro*.

To confirm the interaction of NS1B with Brd2, we performed *in vivo* GST pull down assay (Figure 2.3). The plasmid, pcDNA3-mycBrd2, was co-transfected into 293T cells with a plasmid encoding either GST tagged full length NS1B, the NS1B RBD, or

the NS1B effector domain. As a control, the plasmids encoding only GST or the NS1A protein with N-terminal GST tag were co-transfected with pcDNA3-mycBrd2 into 293T cells. Proteins in extracts from the co-transfected cells were selected on glutathione affinity resins and analyzed by Western blotting.

The Brd2 protein interacts with GST-NS1B (Figure 2.3 upper panel, lane 9) but does not interact with GST and GST-NS1A (Figure 2.3 upper panel, lanes 7 and 8). Furthermore, the Brd2 protein does not interact with the RBD of the NS1B protein (Figure 2.3 upper panel, lane 10) and interacts with the effector domain stronger than with full length protein (Figure 2.3 upper panel, lane 11).

As a next step, in order to test whether the interaction of Brd2 and NS1B is direct, *in vitro* GST pull down assay was performed. Although RNA binding activity of the Brd2 protein has not been reported, we determined whether RNA mediates the *in vitro* interactions, as a control, by including GST-NS1A and GST-NS1B (R50, 53A), an RNA binding defective mutant NS1B protein, in this assay. Furthermore, to confirm if the interaction is mediated by the effector domain of the NS1B protein directly, the GST fused effector domain of the NS1B protein was also used. All GST fused NS1 proteins, used in this assay, are bacterially expressed and purified by using glutathione affinity chromatography.

The *in vitro* translated, [^{35}S] radiolabeled Brd2 protein interacted with GST-NS1B (Figure 2.4 upper panel, lane 2) but it did not interact with GST and GST-NS1A (Figure 2.4 upper panel, lanes 1 and 5). The mutant GST-NS1B (R50, 53A) interacted

●p160 band

# of matched peptide	Name of Identified Protein
13	PININ
7	MBB1A
6	TCOF
3	Q9H6S0
2	O14654
2	AFF4
2	ACINU
2	SMC1A
1	SYIC

●p115 band

# of matched peptide	Name of Identified Protein
19	NOL1
18	BRD2
5	DHX36
3	PARP1
3	DSRAD
3	RBM2
3	BOP1
2	XRN2
2	Q7Z6I0
2	HS105
2	MOV1
2	TCOF
1	Q9NTC3
1	AP3B1
1	BRD3
1	DDX10
1	SND1

●p100 band

# of matched peptide	Name of Identified Protein
24	NUCLEOLIN
15	DDX21
4	UBF1
3	DDX27
2	SRPK1
2	Q7Z6I0

Table 2.1 Result of mass spectrometry Each table includes identified proteins and the number of matched peptide sequences in the protein sequences in each band chosen after double affinity purification (Figure 2.5). Brd2 (red) was chosen for further study because it is a transcriptional regulator with a kinase activity but has not been characterized as an RNA binding protein.

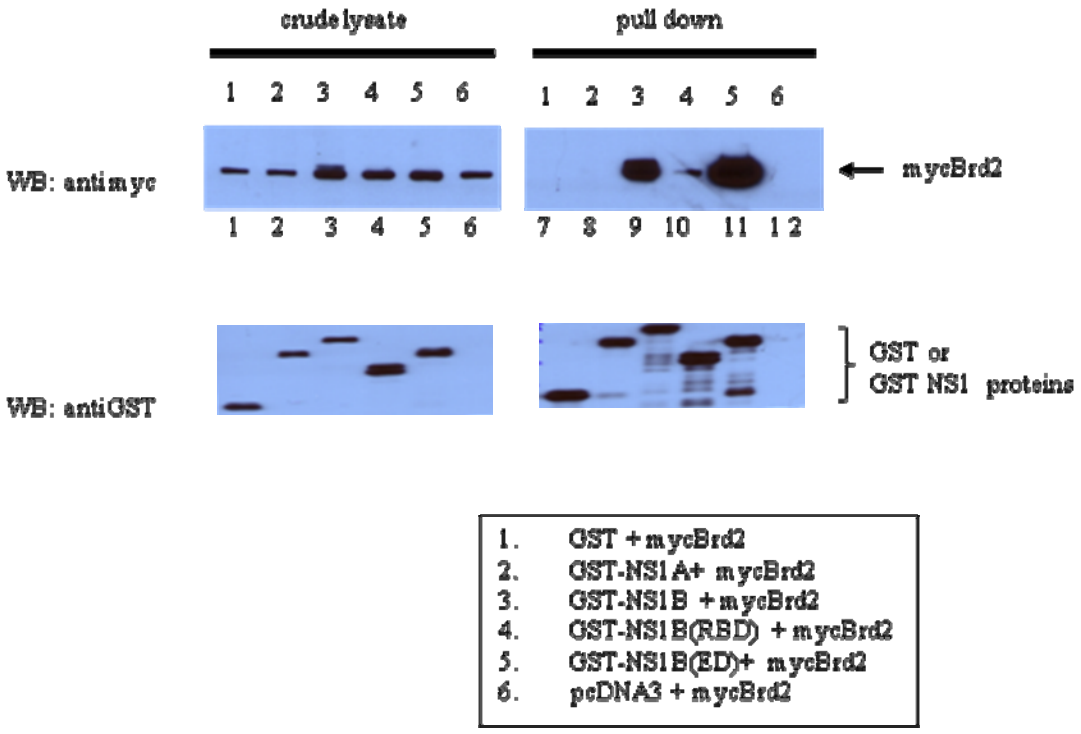

Figure 2.3 NS1B specifically interacts with Brd2 via its effector domain *in vivo*. Proteins in extracts from 293T cells, transfected with the combinations of plasmids which are indicated in the black box, were affinity purified on glutathione sepharose resins and resolved by SDS-PAGE. The purified GST or GST fused proteins were detected by Western blot analysis with goat anti GST antibody (lower panel on right side) and the co-purified myc tagged Brd2 protein were detected with monoclonal anti myc antibody (upper panel on right side). The expression of all transfected proteins in crude lysates was detected by Western blot analysis with antibodies needed (two panels on left side).

with the Brd2 protein (Figure 2.4 upper panel, lane 3) to an extent similar to that of the wild-type protein (Figure2.4 upper panel, compare lanes 2 with 3). Brd2 binding to GST-NS1B (ED) was as strong as that to GST-NS1B (Figure 2.4 upper panel, lane 4). Taken together, these results show that the NS1B protein binds to Brd2 directly that the NS1B effector domain mediates the interaction without RNA-mediation *in vitro* and that the NS1A protein does not bind to Brd2.

Brd2 interacts with the NS1B protein in virus-infected HeLa cells and the interaction domain is comprised of amino acids 146 to 281 of the NS1B protein.

Previous experiments for identification and confirmation of the interaction of Brd2 and NS1B were performed in environment without viral infection. Therefore, we needed to establish the interaction occurred in influenza B virus-infected cells. Interactions in virus-infected cells were determined by co-immunoprecipitation assays. HeLa cells were transfected with the plasmid encoding the N-terminal Flag fused Brd2 protein, followed by infection with various recombinant influenza B viruses encoding full length or C-terminal truncated NS1B. Infected cell extracts were immunoprecipitated with anti Flag M2 agarose affinity resin, and co-immunoprecipitated NS1B proteins in elutes were analyzed by Western blotting.

The NS1B protein interacted with the Brd2 protein in virus infected-cells (Figure 2.5 upper panel, lane 6). As an initial approach to determine the region of the NS1B protein that binds Brd2, two mutant viruses were generated which express either

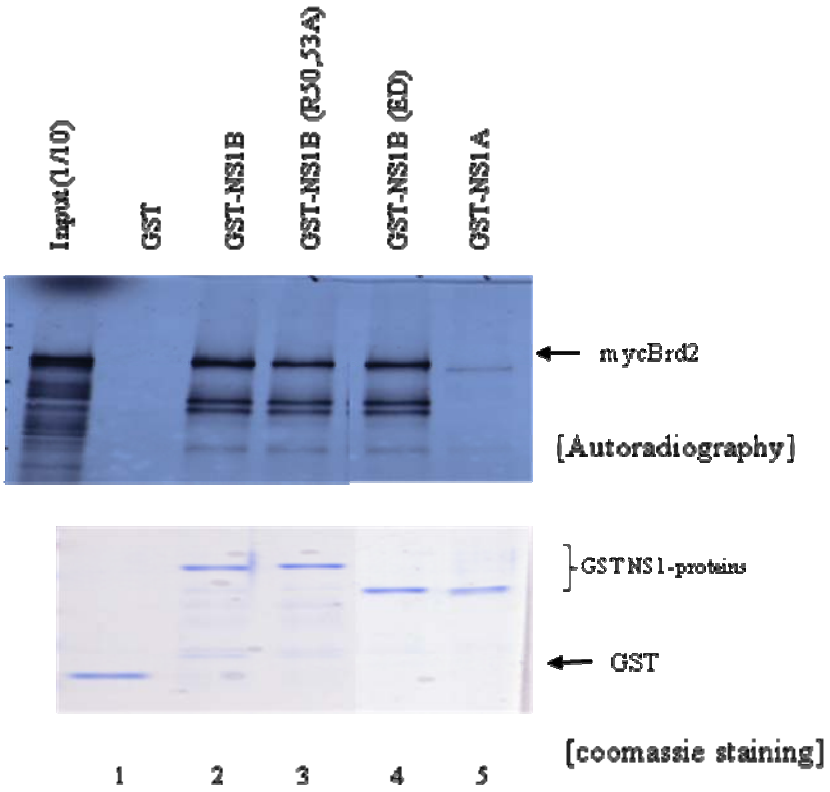

Figure 2.4 NS1B interacts with Brd2 directly and the effector domain of NS1B mediates the interaction *in vitro*. 2μg bacterially expressed and purified GST (lane 1) or GST fused NS1 proteins (lanes 2 to 5) were mixed with *in vitro* translated, [35S] radiolabeled, N-terminal tagged Brd2 proteins and selected on glutathione affinity resin. The affinity-selected and co-selected proteins were resolved by SDS-PAGE. The co-selected Brd2 proteins were detected by autoradiography (upper panel) and the affinity-selected proteins were detected by coomasie blue staining (lower panel). 10% of *in vitro* translated Brd2 proteins were loaded as input control.

the 1-104 amino acid region or the 1-145 region of the NS1B protein. Neither of these truncated NS1B proteins interacted with the Brd2 protein in infected cells (Figure 2.5 upper panel, lanes 7 and 8). These truncated NS1B proteins were expressed as efficiently as the full length NS1B protein (Figure 2.5 lower panel, lanes 3 and 4). These results confirmed the NS1B effector domain-mediated interaction in virus-infected cells and indicated that the binding site of Brd2 on NS1B is a region of amino acids 146 to 281 of the NS1B protein.

DISCUSSION

In present study, we screened cellular proteins which interact with the effector domain of the NS1B protein, which has been reported to be responsible for suppressing production of IFN-β mRNA in virus-infected cells (Dauber et al. 2006; Hai et al. 2008). We tried two affinity purification approaches as described above. As a result of performing the double affinity purification from transfected cells, we identified Brd2 as a cellular protein that specifically interacts with the NS1B effector domain. Based on *in vitro* interaction assay, Brd2 interacts with the NS1B effector domain directly. The cellular interaction of Brd2 and NS1B was confirmed in cells in which both proteins were expressed transiently, or infected with influenza B virus. Furthermore, the Brd2 binding site on the NS1B protein is the region of amino acids 146 to 281 of the NS1B protein.

This is the first case of an interaction between a RNA viral protein and the Brd2 protein. The Brd2 protein is reported as an interacting protein of DNA viral proteins

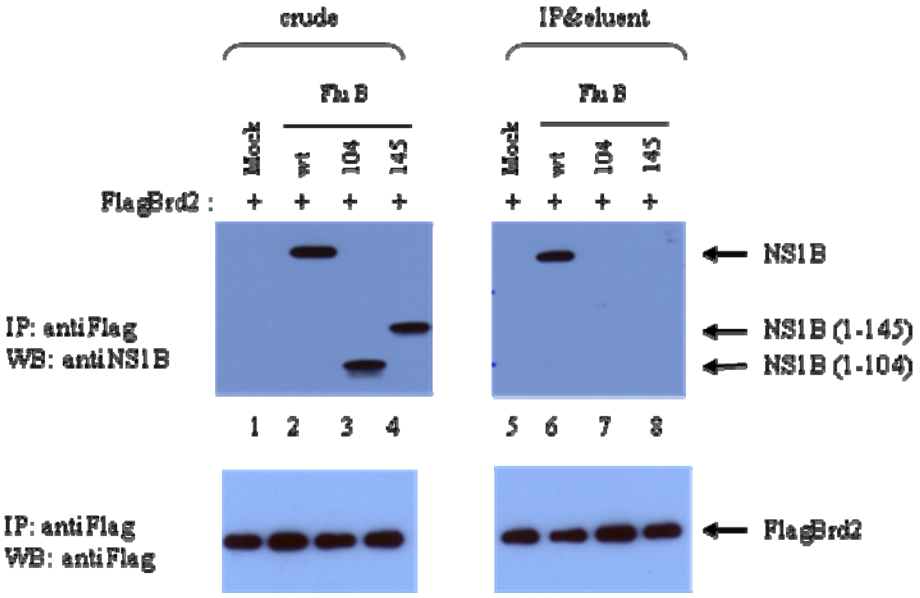

Figure 2.5 NS1B interacts with Brd2 in virus-infected cells and the Brd2 binding site on NS1B can be in C-terminal 136 amino acids of NS1B. HeLa cells were transfected with pCN-FlagBrd2, followed by infection with various influenza B viruses (Flu B) encoding the full length NS1B (wt), the N-terminal 104 amino acids of NS1B (FluB 104), or the N-terminal 145 amino acids of NS1B (FluB 145) at 24 hours post transfection (MOI=10). Cell extracts, prepared at 10 hours post infection, were immunoprecipitated with anti Flag affinity resins and the immunoprecipitated and co-immunoprecipitated proteins were eluted with 3×Flag peptides. Proteins in eluents were resolved by SDS-PAGE, followed by Western blotting using polyclonal anti NS1B antibody to detect the NS1B protein (upper panel on right side) or monoclonal anti Flag antibody to detect the Flag fused Brd2 (lower panel on right side). Proteins in crude lysates were resolved by SDS-PAGE, followed by Western blotting (two panels on left side).

, LANA-1 (latent nuclear antigen) of KHSV (Platt et al. 1999) and the orf73 protein of murine gamma herpes virus 68 (MHV68 orf73) (Ottinger et al. 2009), tethering the viral genome to the mitotic chromosome to evenly segregate viral genomes in virus-infected cells (Ballestas et al. 1999; Moorman et al. 2003). The C-terminal 23 amino acids deletion of LANA-1 abrogates the LANA-1 dependent transactivation of the cyclin E promoter coupled with loss of binding to the Brd2 protein which transactivates several cyclin promoters through associating E2F proteins (Denis et al. 2000; Viejo-Borbolla et al. 2005). MHV68 orf73, a homologue of LANA-1, also interacts with the Brd2 protein. Ottinger et al. determined the Brd2 binding site on the orf73 protein and the binding defective mutant with alanine replacement led to a decreased ability to activate cyclin D1, D2, and E promoters (Ottinger et al. 2009). Taken together, the results suggest that the function of Brd2 binding to DNA viral proteins might be to regulate the transcription of cellular gene. These results raise the question of whether the role of Brd2 binding to the effector domain of NS1B is involved directly with transcription of the IFN-β gene, or whether it functions in other aspects of the activation of IFN transcription, or has any role in IFN production.

We confirmed the interaction of Brd2 and NS1B with extensive interaction assays. Brd2 is confirmed as an interacting protein that only interacts with the effector domain of the NS1B protein but not with NS1A like ISG15 (Yuan and Krug 2001). We will determine the interacting domain of NS1B with the Brd2 protein and the domain will be used as information for reverse genetics and functional study.

CHAPTER THREE

A RECOMBINANT INFLUENZA B VIRUS EXPRESSING A NS1B PROTEIN THAT LACKS THE BINDING SITE FOR THE CELLULAR BRD2 PROTEIN ACTIVATES BOTH IRF3 AND THE SYNTHESIS OF IFN-β MESSENGER RNA

INTRODUCTION

Innate immunity that is induced by interferons (IFNs) in virus-infected cells is the first defense against viral infection. These IFNs include IFN-β and 13 types of IFN-α. Upon influenza virus infection, single-stranded viral RNA with N-terminal tri-phosphate is recognized by a cytoplasmic pattern-recognition receptor (PRR), the retinoic acid inducible gene-I (RIG-I) protein (Kato et al. 2006; Loo et al. 2008). Binding of this viral RNA to the C-terminal domain (CTD) of RIG-I exposes the N-terminal CARD domain, which interacts with the CARD domain of MAVS, an adaptor protein on mitochondria, thereby activating MAVS (Takahasi et al. 2008). Activated MAVS stimulates TBK1, which phosphorylates the C-terminal serine-threonine rich tract of IRF3, constitutively expressed in the cytoplasm (Hemmi et al. 2004; McWhirter et al. 2004). Phosphorylated IRF3 then dimerizes and enters the nucleus, where it associates with CBP/p300, a transcriptional co-activator. The complex of the phosphorylated IRF3 and CBP/p300

combines with activated NF-κB and AP-1 to form the enhanceosome which binds to the IFN-β promoter and turns on IFN-β transcription. IFN-β induces the transcription of IFN-stimulated genes (ISGs), some of which encode antiviral proteins (Chapter one, Figure 1.6).

Viruses have evolved various mechanisms to inhibit the production of IFN-β in virus-infected cells. Several viruses trim 5' end tri-phosphate of viral RNA that would activate RIG-I (Habjan et al. 2008). Ebola virus VP35 protein and Vaccinia virus E3L protein sequester dsRNA from PRRs (Xiang et al. 2002; Cardenas et al. 2006). RIG-I is directly targeted by some viral proteins. The NS2 protein of RSV (respiratory syncytial virus) binds to RIG-I and prevents RIG-I from associating with MAVS (Ling et al. 2009). Mda5, another cytoplasmic PRR, is targeted by the V proteins of several paramyxoviruses which bind to Mda5, resulting in inhibiting the self-association of Mda5 (Childs et al. 2009). The cleavage and then release of MAVS from mitochondria by viral proteases of picornaviruses including HCV, HAV, and GB virus B ablates the induction of IFN-β in the virus-infected cells (Meylan et al. 2005; Chen et al. 2007; Yang et al. 2007). The activation of TBK1 is blocked by direct association of the P protein of borna disease virus (BDV) (Unterstab et al. 2005) or rabies virus (Brzozka et al. 2005).

Viral proteins target the activation of IRF3 by several mechanisms. The V proteins of paramyxoviruses are phosphorylated by TBK1 as pseudosubstrates, and thereby interfere with the subsequent activation of IRF3 (Lu et al. 2008). The papain-like protease domain (PLpro) of severe acute respiratory syndrome coronavirus (SARS-CoV) directly binds to IRF3 and inhibits IRF3 phosphorylation (Devaraj et al. 2007).

Some viral proteins cause proteasome-mediated degradation of IRF3 via their interaction with IRF3 (Barro and Patton 2005; Bauhofer et al. 2007; Saira et al. 2007). The viral IRF1 (vIRF1) protein of herpes simplex virus blocks the association between activated IRF3 and CBP/p300 (Lin et al. 2001). A viral transcription factor, the K-bZIP protein of KHSV, competes with IRF3 for binding to the IFN-β promoter, thereby suppressing the activation of IFN-β transcription (Lefort et al. 2007).

Influenza virus also suppresses IFN-β synthesis in virus-infected cells. The NS1 proteins of influenza A and B viruses are responsible for the suppression. The NS1 protein comprises two domains: a RNA binding domain (RBD) encompassing the N-terminal one third of the NS1 protein and an effector domain containing its remaining part. The effector domain of the NS1 protein suppresses IFN-β synthesis. The NS1 protein of most human influenza A viruses (the NS1A protein), inhibits the 3'-end processing of IFN−β pre-mRNA, as well as of other cellular pre-mRNAs (Nemeroff et al. 1998; Das et al. 2008). In contrast, the NS1 protein of influenza B viruses (the NS1B protein) suppresses the transcription of the IFN−β gene. The increased IFN−β production in cells infected with a recombinant virus encoding an NS1B protein lacking the effector domain of NS1B demonstrated that the NS1B effector domain is responsible for the suppression (Dauber et al. 2006). However, the mechanism by which the effector domain of the NS1B protein suppresses IFN−β mRNA synthesis in virus-infected cells has not been elucidated.

The Brd2 protein is a member of BET family proteins which have two bromodomains (BDI and BDII) that bind acetylated histones (Kanno et al. 2004; Loyola

and Almouzni 2004), and an extraterminal (ET) domain (Wu and Chiang 2007) that mediates protein-protein interactions (Platt et al. 1999; Denis et al. 2000; You et al. 2004; Ottinger et al. 2006; You et al. 2006; Ottinger et al. 2009). Brd2 was originally identified as a mitogen activated nuclear kinase (Denis and Green 1996), and is highly conserved in numerous species (Thorpe et al. 1996). Brd2 localizes throughout the cell in resting cells, whereas mitogen treatment induces its nuclear localization (Guo et al. 2000). The nuclear Brd2 protein facilitates the transcription of genes in euchromatin via its association with hyperacetylated histones (LeRoy et al. 2008), and synergistically enhances E2F1-dependent gene transcription via its association with E2F1 (Denis et al. 2000). In contrast, the function of the cytoplasmic Brd2 in resting cells has not been elucidated.

Two DNA viral proteins, the LANA-1 protein of KHSV and the orf73 protein of MGHV68, have been identified as Brd2-binding viral proteins. These proteins tether viral genomes to the cellular chromatin to facilitate the equal segregation of viral genomes during their latent infection (Ballestas et al. 1999; Moorman et al. 2003). The Brd2 binding ability of LANA-1 or the orf73 protein regulates transactivation of cellular genes. The deletion of Brd2 binding site on the LANA-1 protein led to the downregulation of the LANA-1-dependent transactivation of the cyclin E promoter (Denis et al. 2000; Viejo-Borbolla et al. 2005). Similarly, alanine replacement mutations, introduced into both Brd2 and Brd4 binding site on the orf73 protein, compromise promoter transactivation of several cyclin-genes such as cyclin D1, D2, and E (Ottinger et al. 2009). Those viral proteins require the Brd2 binding activity for their transactivation of cellular genes.

The previous screen for a cellular protein that binds to the NS1B effector domain led us to identify the Brd2 protein as a specific binding protein of the NS1B effector domain. In this study, binding assay using several truncated NS1B proteins led us to identify the NS1B region extending from amino acids 171 to 190 of NS1B as the Brd2 binding domain on NS1B. Further mutagenesis experiments described here pinpointed the phenylalanine at position 171 (F171) of NS1B as the essential amino acid within the Brd2 binding domain on NS1B. We took a reverse genetic approach to study the role of the Brd2 binding activity of NS1B in viral replication and in antiviral response. Analysis of a recombinant virus encoding an NS1B protein lacking its Brd2 binding activity demonstrated that the Brd2 binding activity of NS1B is required for suppressing IRF3 activation and IFN-β mRNA synthesis. The increased IFN-β mRNA in cells infected with the mutant virus caused attenuation. To determine if Brd2 mediates the suppression in infected cells, we carried out IRF3 dimerization assay after downregulating endogenous Brd2 proteins using the siRNA or shRNA against Brd2 but the approach did not give us clear answer (see below). Furthermore, binding analysis using other Brd family proteins demonstrated that Brd4, a functional redundant protein, also interacts with the NS1B protein, indicating that siRNA-knockout of Brd2 alone would likely not be sufficient to restore wild-type phenotype

MATERIALS AND METHODS

Cell lines HEK293 (human embryonic kidney cells), HEK293T (human embryonic kidney cells), HEL299 (human embryonic lung cells), MDCK (Madin-Darby canine kidney cells), HeLa (human cervical cancer cells), and HeLa-tet-on (human cervical cancer cells) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (compete DMEM media) at 37°C under a 5% CO_2 atmosphere. CaLu-3 (human fetal lung epithelial cells) were cultured in Advanced MEM (Invitrogen) supplemented with 10% FBS and antibiotics at 37°C under a 5% CO_2 atmosphere.

Expression vectors To generate GST tagged NS1B mutants, DNA fragments corresponding to residues, amino acids 94-170, 94-190, 94-281, 136-190, and 168-190 of NS1B were amplified by PCR with primers containing restriction sites Not I and Xba I (Figure 3.3 A). The PCR products were cloned in-frame into a GST tag containing vector (pCN-GST). Five original amino acids 171 to175, 176 to 180, 181 to 185, or 186 to 190 of NS1B were replaced by a tract of 5 alanines by two round of PCR with adequate primers (Figure 3.4 A). Five alterations of NS1B original amino acids to single alanine within amino acids 171 to 175 of NS1B were performed by using two rounds of PCR and specific primers. The resulting DNA was sequenced and cloned in-frame into the pCN-GST vector or pAD3000 vector to generate recombinant viruses expressing mutant NS1B proteins.

Generation of a recombinant influenza B/Yamanashi/166/98 virus by transfecting 8 plasmids 293T cells, transiently transfected with 8 plasmids encoding the full-length

cDNAs for each of eight influenza B/Yamanashi/166/98 viral genomic sequences, were overlaid on subconfluent MDCK at 16 hours post transfection. At various hours post transfection, culture supernatants were collected as previously described (Hoffmann et al. 2002). Recombinant viruses encoding each point-mutant NS1B protein having replacement of F171, R172, L173, T174, or I175 of NS1B with alanine were generated by replacing the pA3000 vector expressing the wild type NS gene with that expressing each NS gene containing each point mutation. The generated viruses were tittered by plaque assay on MDCK cells and individual plaques were amplified in 10-day-old embryonated chicken egg. The amplified viruses were tittered by plaque assay. Viral RNA segments were sequenced. Some viruses were amplified in MDCK cells after multiple cycle infection.

Plaque assay, multiple cycle infection, and determination of a cellular interaction using GST pull down assay We followed the protocol as described in same section of chapter two. Multiple cycle infection was preformed in Calu-3 cells instead of in MDCK cells because the Calu-3 cells were recently determined as a human cell where influenza viruses grow efficiently (Tumpey et al. 2005).

Quantitative real time RT-PCR 293 cells were infected mock, wild type, or mutant F171A virus at a moi of 3. Total RNA from the infected cells were extracted with TRIzol reagent (Invitrogen) at 6 hours post infection. For each sample, 5μg of total RNA corresponding to equal cells equivalents, were reveres transcribed (Superscript first

strand kit, Invitrogen) using oligo dT. The amount of IFN-β mRNA was determined using the TagMan Gene Expression Assay (Applied Biosystems) with primers complementary to internal sequence of IFN-β mRNA (Forward: TGC ACC TGA AGA AAT ATT AC; Reverse: GAT TTC TGC TTG GAC TAT TGT CCA GG) and a 6-FAM dye-labeled TaqMan MGB (minor groove binder) internal probe. Real time PCR analysis was carried out using the Perkin-Elmer/Applied Biosystems 7900HT Sequence Detector.

Co-immunoprecipitation assay to test the IRF3 association with CBP in virus-infected cells 293 cells on 10cm plate were transfected with 10μg of a plasmid encoding Flag tagged IRF3 (pcDNA3-FlagIRF3) and were infected with mock, wild type, or mutant F171A virus at a moi of 5 at 24 hours post transfection. The prepared extracts from the infected cells at 8 hours post infection were immunoprecipitated with anti Flag M2 affinity agarose resins. Immunoprecipitated and co-immunoprecipitated proteins were resolved on a SDS-polyacrylamide gel. To detect immunoprecipitated proteins, proteins on gel were transferred on nitrocellulose by semi dry transfer method but, for detecting co-immunoprecipitated proteins, proteins were transferred on PVDF membrane at 75V overnight by tank transfer method in cold room. The co-immunoprecipitated endogenous CBP proteins were detected by Western blotting assay using monoclonal anti CBP antibody (A-22, Santa Cruz).

IRF3 dimerization assay to measure the activation of endogenous IRF3 Confluent 293 cells or HEL299 cells on 6-well plate were infected with influenza A/Udorn/72 virus,

wild type or mutant influenza B viruses as indicated. The infected cells were resuspended in 120µl of lysis buffer (50mM Tris·HCl (pH7.5), 150mM NaCl, 1mM EDTA (pH8.0), 1%NP40, 1X complete protease inhibitor cocktail (Roche), 1mM Na3VO4, 1mM β-glycerophosphate, 50mM NaF), vortexed, incubated for 10 minutes on rotation platform in cold room, and centrifuged for 5 minutes at 10,000×g. Aliquots of 10µg protein were resolved on 7.5% native polyacrylamide gel with 1% deoxycholate (DOC) in the cathode buffer as described (Tomokatsu Iwamura et al. 2001). IRF3 monomers and homodimers were detected by Western blot analysis using polyclonal anti IRF3 antibody (FL-425, Santa Cruz). Virus infections were determined by detecting NS1 proteins with polyclonal anti NS1A or NS1B antibody after resolving proteins in whole cell lysates on a SDS-polyacrylamide gel and transferring on nitrocellulose membrane.

siRNA interference To knock down endogenous Brd2 in 293 cells or HeLa-tet-on cells, ON-TARGET plus smart plus siRNA for human Brd2 (L-004935, Dharmacon) (siBrd2) was used. As a control, ON-TAGET plus non-targeting pool (D-001810, Dharmacon) siRNA (control siRNA) was used. Confluent HEK293 or HEL299 cells on 10cm plate were seeded on each well of a 6-well plate at 1:40 dilution at 24 hours before siRNA transfection. HEL299 cells were transfected with 100nM of siBrd2 or control siRNA using Xtreme siRNA transfection reagent (Roche) and culture media containing the siRNA-transfection reagent complexes were changed by replenishing fresh media at 8 to 12 hours post transfection. To knock down cellular Brd2 in 293 cells, 100nM of the indicated siRNA were transfected into the cells using Dharmafect I transfection reagent

(T-2001, Dharmacon) by following manufacturer's protocol. The transfected HEK293 or HEL299 cells were infected with same set of viruses at 3 days post transfection. Extracts were prepared with the infected cells at 6 hours post infection and followed by SDS-PAGE. The knock down of endogenous Brd2 was verified by Western blotting analysis using goat anti Brd2 antibody (A300-204A, Bethyl Laboratories; ab3718, Abcam). IRF3 dimerization assay was carried out with another set of extracts prepared from cells treated in duplicate.

shRNA interference To generate a plasmid encoding short-hairpin RNA (shRNA) targeting Brd2 mRNA, eleven target sequences were chosen among sequences, predicted by the prediction program provided by Dharmacon or Ambion. We ordered eleven sets of two complementary oligonucleotides with single-stranded overhangs complementary to the overhangs from the restriction enzyme site in the multi cloning site: BamH I and Hind III. These were annealed, phosphorylated, and ligated into the pSilencer 5.1 H1 retro vectors (Ambion). 293 cells were transfected with one of 11 generated plasmids and at 3 days post transfection, endogenous Brd2 proteins in the transfected cells were detected by Western blotting analysis. Verified plasmids encoding shRNA to deplete endogenous Brd2 proteins were used for further experiments. The production of IFN-β mRNA was detected by RT-PCR in cells transfected with a verified plasmid to encode shRNA, followed by infection of the indicated viruses at a moi of 3 for 6 hours.

Luciferase assay 293T cells were seeded at 50% confluency on 24-well plate. At next day, cells were co-transfected with a mixture of plasmids including 100ng of plasmids encoding fire fly luciferase under control of the INF-β promoter (pIFNβpL) and 25ng of plasmids expressing renilla luciferase under the control of TK promoter (pRL) as internal control together indicated amounts of plasmids encoding proteins using Trans IT LT1 transfection reagent. The luciferase activities in lysates from the transfected cells were measured with luminometer at 48 hours post transfection. All experiments were performed in duplicate and activities of fire fly luciferase were normalized to those of renilla luciferase. The dual luciferase assay kit (Promega) was used for this assay.

RESULTS

The region of NS1B extending from amino acids 168 to 196 is necessary and sufficient for binding Brd2.

To determine the Brd2 binding domain on the NS1B effector domain, we measured the binding of N-terminal myc-tagged Brd2 with N-terminal GST fused NS1B proteins containing either the whole effector domain or the amino acids 94-170 or 94-190 (Figure3.3 A). Extracts from cells transfected with two plasmids encoding the myc tagged Brd2 and one of the truncated NS1B mutants were subject to affinity selection on glutathione sepharose resin. The co-purified myc fused Brd2 proteins were detected by Western blotting analysis. As shown in Figure 3.1, Brd2 interacts with the NS1B proteins

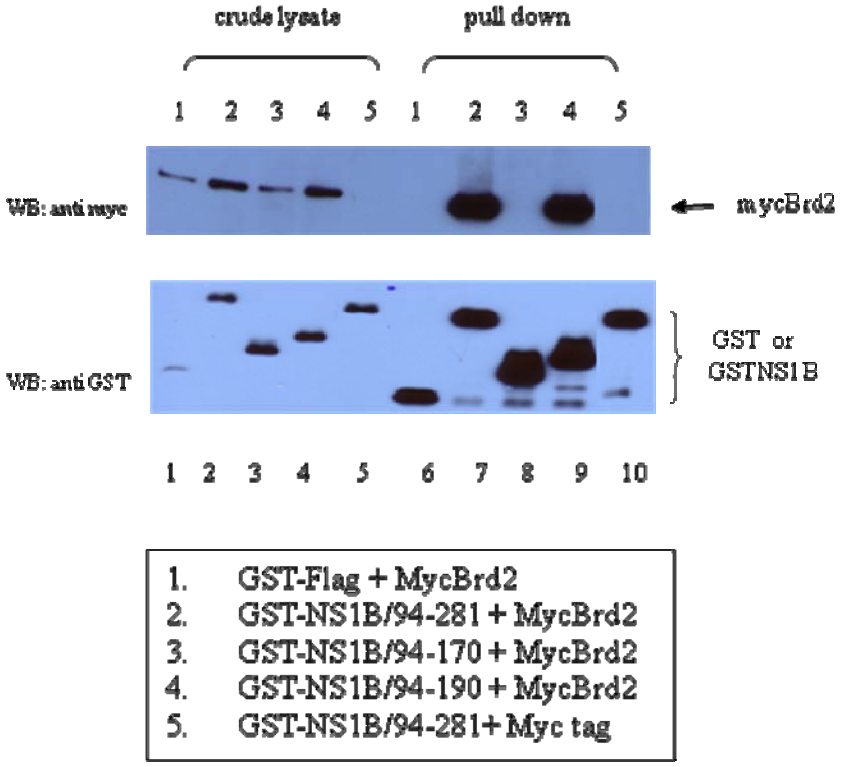

Figure 3.1 Amino acids 171 to 190 of NS1B are essential domain for the Brd2 binding on NS1B. Extracts from cells, transfected with sets of plasmids encoding the proteins as shown in black box, were pulled down by glutathione sepharose. Both affinity-purified and co-purified proteins on the resins were resolved by SDS-PAGE. The GST fused NS1B proteins were detected by Western blotting using anti GST antibody (lower panel, lanes 6 to 10) and the co-purified myc tagged Brd2 proteins were detected by Western blotting analysis using monoclonal anti myc antibody (upper panel, lanes 6 to 10). The expression of proteins in extracts was determined by Western blotting using the antibodies to detect indicated tags (lanes 1 to 5 of both panels).

containing the effector domain (NS1B/ 94-281) and amino acids 94 to 190 of NS1B (NS1B/94-190) to a similar extent (Figure 3.1 upper panel, lanes 7 and 9). However, the NS1B protein including amino acids 94-170 (NS1B/94-170) did not interact with Brd2 (Figure 3.1 upper panel, lane 8). The NS1B/94-170 protein was expressed as much as the other NS1B mutants (Figure 3.1 lower panel, compare lanes 3 with 2 and 4), and the amount of pulled-down NS1B/94-170 protein was similar to that of the others (Figure 3.1 lower panel, compare lanes 8 with 7 and 9). These results indicate that amino acids 171 to 190 of NS1B are necessary for binding Brd2.

To identify the domain of NS1B that is sufficient for interacting with Brd2, we employed two N-terminal GST fused, truncated NS1B proteins containing either amino acids 136-190 or amino acids 168-196 (NS1B/136-190 or NS1B/168-196) in this assay. Both NS1B domains bound Brd2. (Figure 3.2 upper panel, lanes 7 and 8). Because the NS1B/168-196 protein (Figure 3.2 lower panel, lane 4) was not expressed as well as either the full-length NS1B effector domain (NS1B/94-281) or NS1B/136-190 (Figure 3.2 lower panel, lanes 2 and 3), its activity in binding Brd2 was comparable to that of the full-length effector domain. We can conclude that the NS1B region comprised of amino acids 168 to 196 is sufficient for binding Brd2.

The Brd2 binding domain on NS1B is highly conserved.

We collected 372 full length NS1B protein sequences from the site http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html and performed multiple sequence alignment analysis. The Brd2 binding region on NS1B extending from amino acids 171

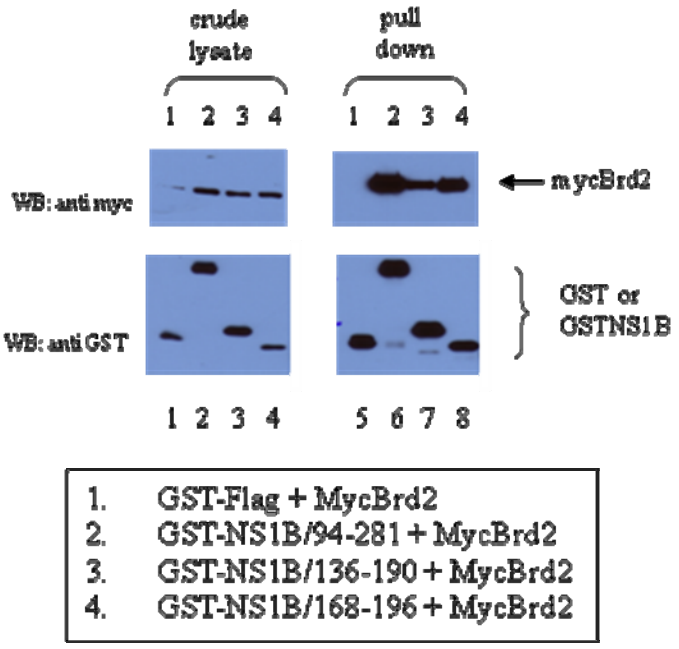

Figure 3.2 Amino acids 168 to 196 of NS1B are sufficient for binding Brd2. The GST or GST fused NS1B truncated mutants in extracts from 293T cells, transfected with two plasmids encoding one of the denoted proteins in the black box, were pulled down with glutathione sepharose affinity resin. The purified GST or the GST fused, truncated NS1B proteins (lower panel, lanes 5 to 8) and co-purified myc tagged Brd2 proteins (upper panel, lanes 5 to 8) were detected by Western blotting analysis using antibodies as needed. Proteins in crude lysates were detected by Western blotting using antibodies as needed (upper and lower panels, lanes 1 to 4).

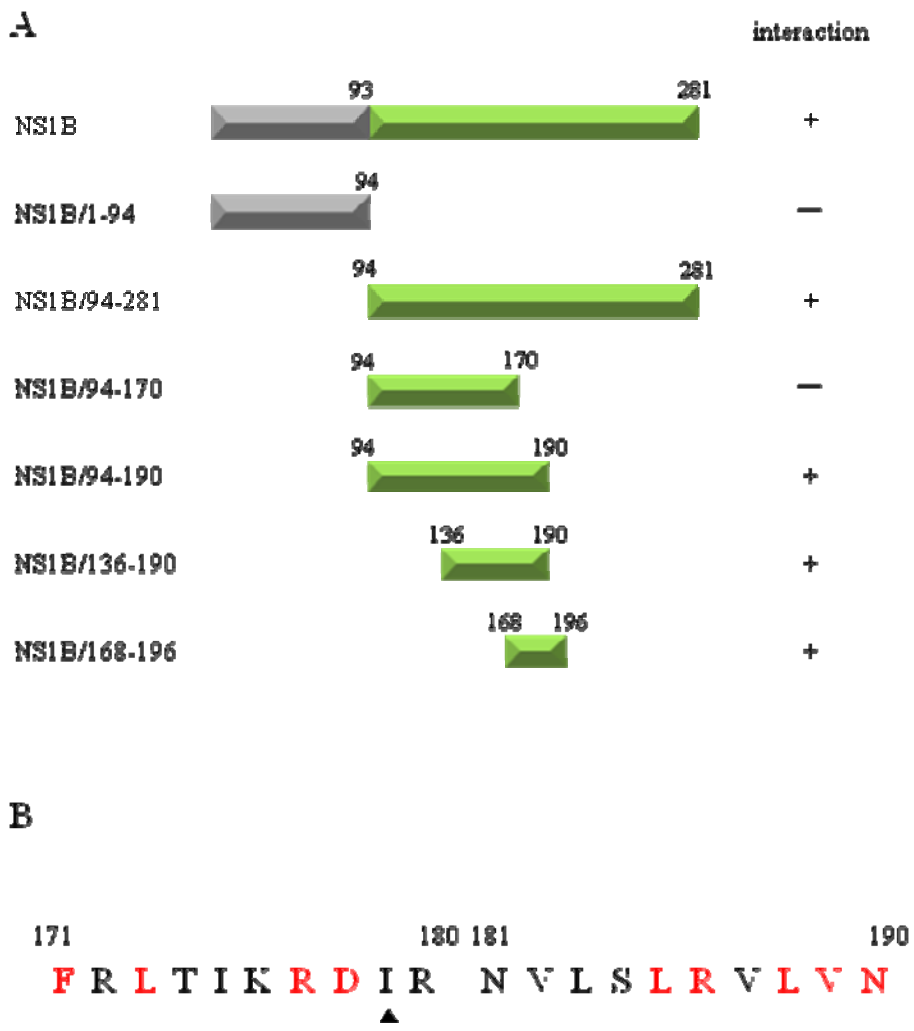

Figure 3.3 Summary of mapping the essential Brd2 binding domain on NS1B and protein consensus sequences of the domain (A) Diagrams represent the NS1B fragments expressed as fusion proteins. The ability of interaction with Brd2 is summarized on the right. The RBD (gray) and the effector domain (green) of the NS1B protein are indicated. The amino acids of NS1B are denoted as numbers. **(B)** The consensus amino acid sequences were determined by multiple alignment analysis with the NS1B protein sequences from 372 influenza B virus strains. 9 of 20 amino acids are completely conserved (written in red color). Except for isoleucine at position 179 of NS1B (marked with black arrow head), the variability of the other sequences are less than 2%. The amino acids of N1B are denoted numbers above.

to 190 is highly conserved among influenza B virus strains (Figure 3.3 B). The variability of each position is less than 2%, except for position 179 which varies about 40% (Figure 3.3 B, black arrow head). Interestingly, the consensus isoleucine at position 179 of the NS1B protein has been replaced with methionine since 2002. The amino acid sequences of the Brd2 binding domain on the NS1B protein of influenza B/Yamanashi/166/98 virus are completely matched with the protein consensus sequences of the domain as shown in Figure 3.3 B.

The phenylalanine at position 171 (F171) of the NS1B protein is the essential amino acid for Brd2 binding

To narrow down the Brd2 binding site on the NS1B protein, we employed several NS1B mutants with replacement of original amino acid(s) in the binding domain by alanine(s). We tested interactions by repeating the GST pull down assay as previously described using extracts expressing the myc tagged Brd2 and one of the GST-tagged mutant NS1B proteins.

As first, we generated four NS1B mutants replacing 5 original amino acids with 5 alanines as described in Figure 3.4 A. Brd2 binding to the NS1B 171/5A protein was completely abolished (Figure 3.4 B upper panel, lane 10). Brd2 binding was retained by the 176/5A and 181/5A proteins (lanes 12 and 13 on upper panel), and a darker exposure of this gel showed that the 186/5A protein retained binding activity (lane 14 on upper panel). The Brd2 interactions with the NS1B mutants were significantly weaker than that with wild type NS1B (Figure 3.4 B upper panel, compare lanes 12 to 14 with 9). It is

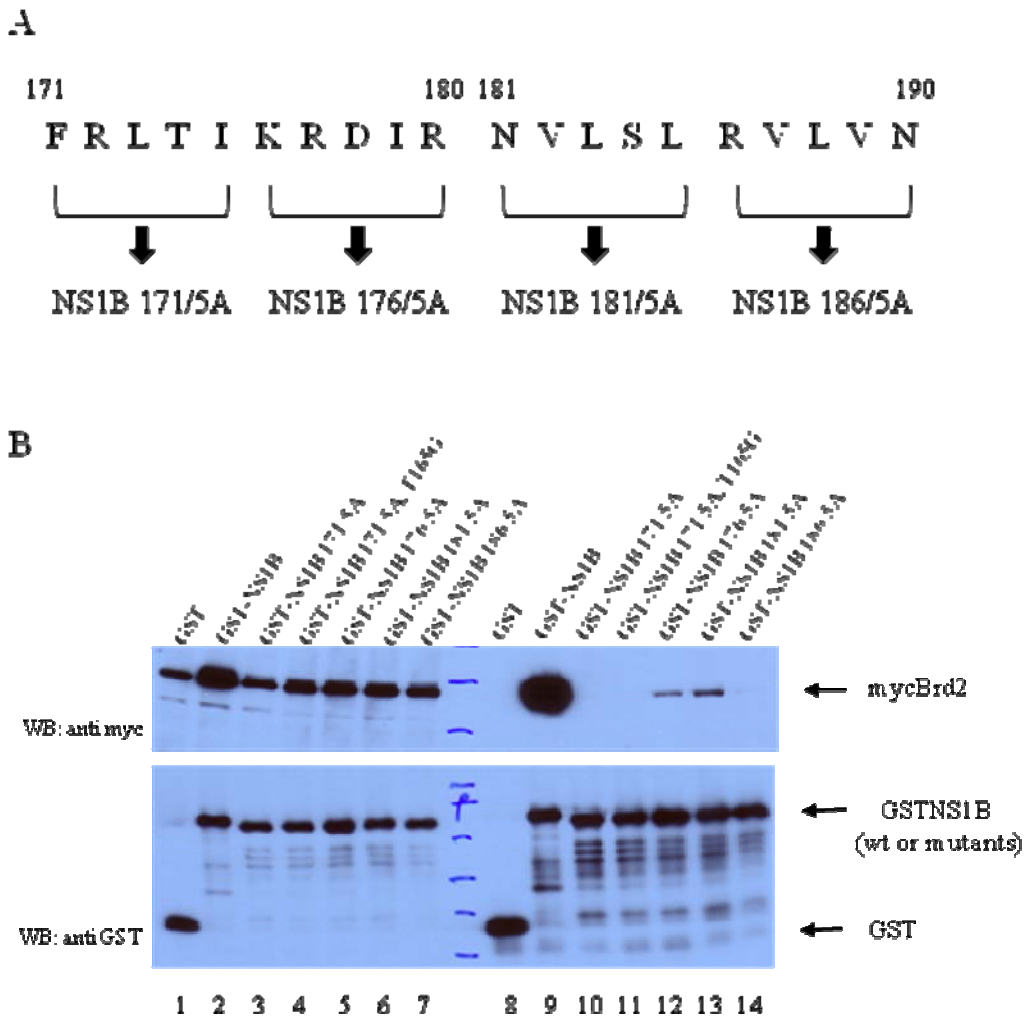

Figure 3.4 Amino acids 171 to 175 of NS1B are essential for the interaction between Brd2 and NS1B. (A) The amino acid sequences at position from 171 to 190 of NS1B are shown. Five sequences, denoted by brackets, were replaced with five alanines and the name of the NS1B mutant proteins were indicated by arrows. The number on the amino acids indicates the position in NS1B. **(B)** Proteins in crude lysates from 293T cells, co-transfected with plasmid encoding the N-terminal tagged Brd2 and one of plasmids encoding GST or GST fusion NS1B proteins, indicated on the upper panel, were selected on glutathione sepharose affinity resin and the purified GST or GST fusion proteins (lower panel lanes 8 to 14) and co-purified Brd2 proteins (upper panel lanes 8 to 14) were detected by Western blotting using the antibody as needed. Proteins in crude lysates were subjected to Western blot analysis (upper and lower panel, lanes 1 to 7).

likely that that replacement of five alanines may causes structural defects in the NS1B protein which lead to reduced binding. Nevertheless, the completely ablated Brd2 interaction with the NS1B 171/5A protein suggested that amino acids 171 to 175 of NS1B are important for the Brd2 interaction.

As next step, we replaced each amino acid at positions 171 to 175 of NS1B with an alanine and those are termed as NS1B F171A, NS1B R172A, NS1B L173A, NS1B T174A, and NS1B I175A. Neither the NS1B F171A protein (Figure 3.5 panel1, lane 4) nor the NS1B 171/5A protein (Figure 3.5 panel 1, lane 3) showed a detectable interaction with Brd2. The expression level of the NS1B F171A protein was similar to that of the other NS1B mutants or wild type NS1B proteins (Figure 3.5 panel 4) and the amount of the NS1B F171A protein in the pulled-down fraction was as much as that of the other NS1B proteins in pull-down fractions (Figure 3.5 panel 3). The NS1B T174A protein interacted with Brd2 to a extent similar to the wild type NS1B protein (Figure 3.5 panel 1, compare lanes 2 with 8), whereas the other L172, R173 and I175 mutants showed significantly weaker binding (Figure 3.5 panel 1, lanes 5 to 7). The completely abolished interaction of Brd2 and the NS1B F171A protein suggested that the phenylalanine at position 171 (F171) of NS1B is the essential amino acid in the binding domain.

To determine the effect of these single amino acid substitutions on virus replication, we generated five recombinant viruses encoding NS1B F171A, NS1B R172A, NS1B L173A, NS1B T174A, or NS1B I175A instead of the wild type NS1B protein. All mutant viruses were successfully generated and they were named as F171A virus, R172A virus, L173 virus, T174A virus, and I175A virus (Figure 3.6). The plaques

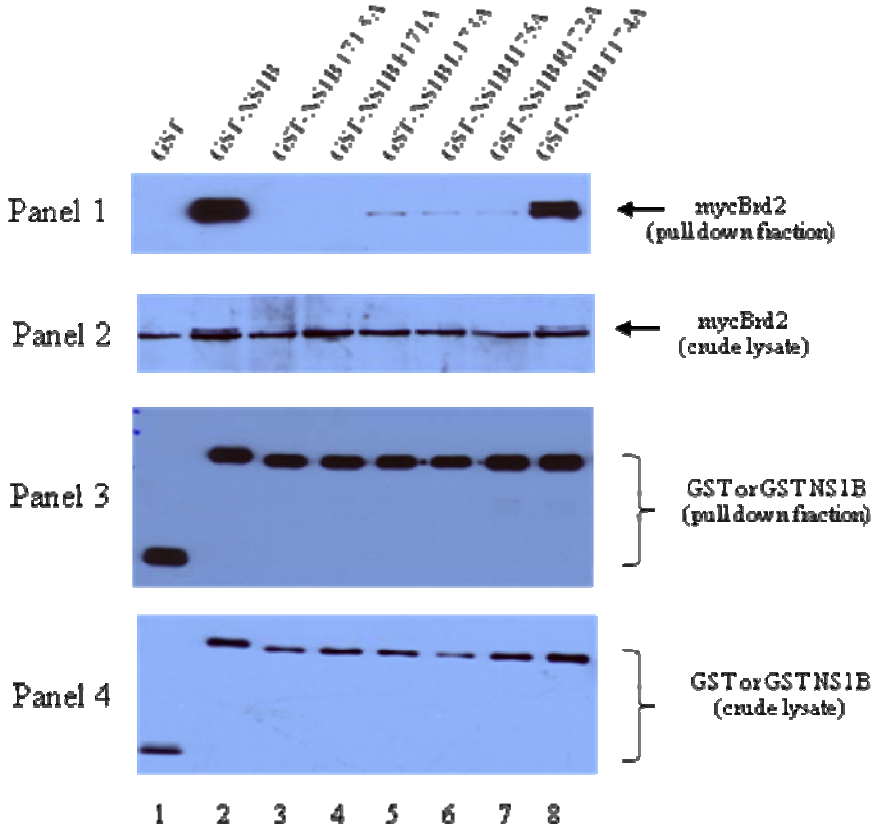

Figure 3.5 The phenylalanine at position 171 of NS1B is the absolute essential amino acid for the interaction. 293T cells in which both the myc tagged Brd2 and one of the NS1B proteins, indicated above panel 1, were expressed by transient transfection. Extracts from the transfected cells were subjected to the GST pull down analysis. GST or GST fused protein in the pull-down fraction (panel 3) or in the crude lysates (panel 4) were detected by Western blotting analysis. Myc tagged Brd2 in pulled down fraction (panel 1) or in crude lysate (panel 2) were analyzed by Western blotting.

in MDCK cells infected with the F171A virus were smaller than those in MDCK cells infected with wt virus or the other mutant viruses, although the size of plaques were heterogeneous (Figure 3.6). The similar size plaques in cells infected with T174A virus to those in cells infected with three other mutant viruses encoding the mutant NS1B proteins having significant weak binding activity to Brd2 (Figure 3.5) demonstrated that even weak interaction between Brd2 and NS1B is sufficient for efficient viral replication. Taken together the results of the GST pull down assay, F171 of NS1B is essential for both the efficient viral replication and the interaction with Brd2. Interestingly, the residue F171 of NS1B is one of the complete conserved amino acid sequences in the region of amino acids 171 to 190 of NS1B (Figure 3.3 B).

A recombinant influenza B virus encoding the NS1B protein with replacement of F171 of NS1B with alanine (F171A virus) is attenuated in Calu-3 cells.

Based on the previous reverse genetics, we can determine the attenuation of F171A virus by plaque assay (Figure 3.6). As next, we assessed the degree of attenuation accurately. We employed Calu-3 cells, human lung fetal epithelial cells, in this assay because influenza B virus is almost exclusive human virus. Moreover, the efficient multiple cycle growth of influenza B virus in Calu-3 cells was determined in our laboratory. Calu-3 cells were infected with wt virus or F171A virus as a moi of 0.001 and amount of viruses in collected culture supernatants was determined by plaque assay (Figure 3.7 A). The yield of the F171A virus replication was 40~50-fold lower than that

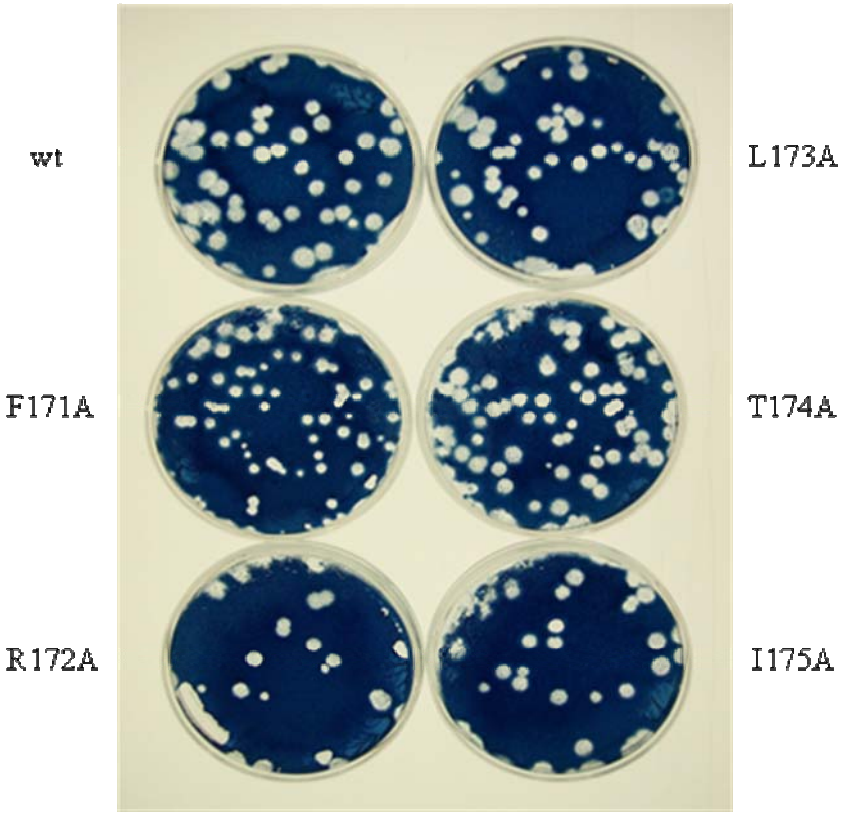

Figure 3.6 The plaques in MDCK cells infected with F171A virus were smaller than those in MDCK cells infected wt or the other mutant viruses. MDCK cells were infected with wild type or mutant viruses encoding a mutant NS1B protein with replacement an amino acid at position from 171 to 175 of NS1B by single alanine The infected cells were overlaid with DMEM containing 1% agarose, 1μg/ml NAT, and antibiotics after 1 hour adsorption and stained after 4-day incubation at 34°C under an atmosphere of 5% CO_2.

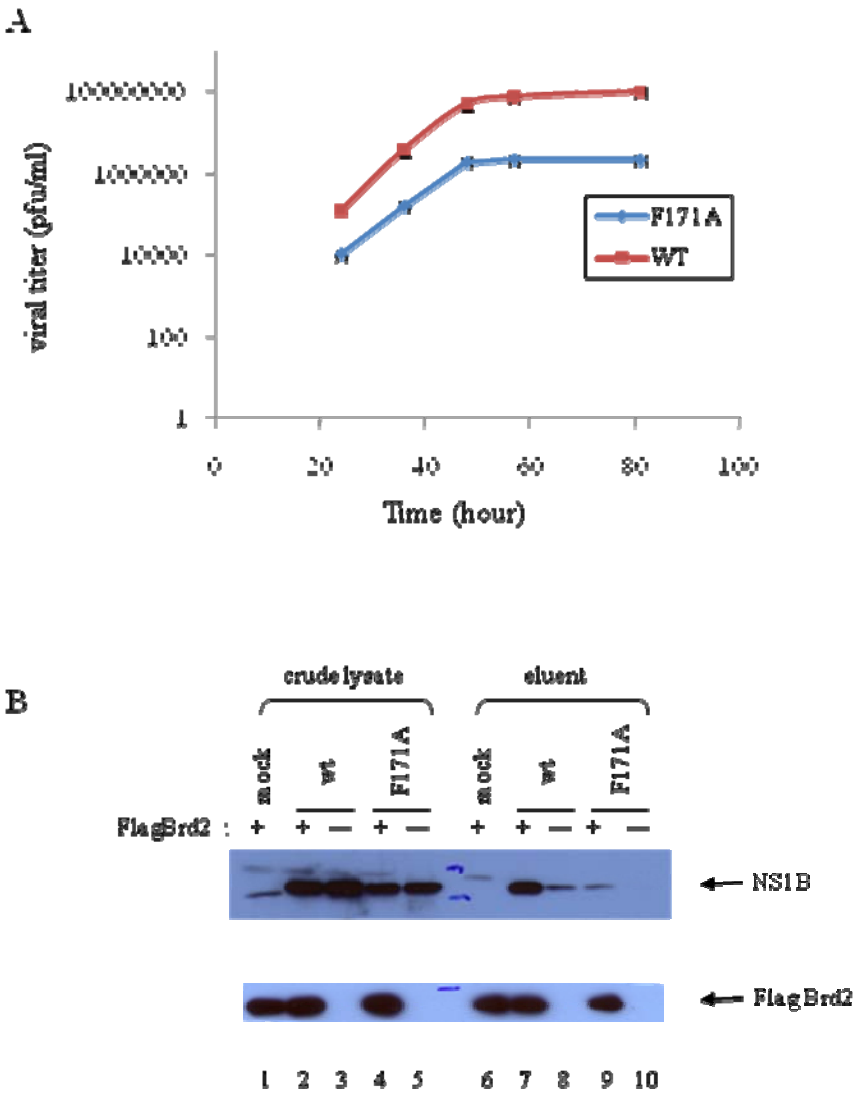

Figure 3.7 F171A virus is attenuated in Calu-3 cells and the mutant NS1B F171A protein does not interact with Brd2 in HeLa cells infected with F171A virus. (A) Calu-3 cells were infected with either wt or F171A virus at a moi of 0.001 and supernatant of cultured cells were collected every 12 hours. The amount of virus in the supernatants was determined by plaque assay on MDCK cells. **(B)** HeLa cells, transfected with the plasmid encoding the N-terminal Flag tagged Brd2 protein (FlagBrd2), were infected with wt, or F171A virus at a moi of 10 at 24 hour post transfection. Extracts from the infected cells were immunoprecipitated with anti Flag M2 agarose resin. Bound proteins were eluted with 3×Flag peptide. The FlagBrd2 in crude lysate (lower panel lanes 1 to5) and in eluents (lower panel lanes 6 to 10) were detected by Western blotting. NS1B proteins in crude lysate (upper panel, lanes 1 to 5) and in eluents (upper panel, lanes 6 to 10) were subjected to Western blot analysis.

of wt virus. Wt virus replicated ~2 fold faster than F171A virus at early time point. Based on these data, we confirmed the attenuation of F171A virus in human cell lines.

The mutant NS1B F171A protein does not interact with Brd2 in cells infected with F171A virus.

To correlate the ablation of the NS1B-Brd2 interaction with the attenuation of the F171A virus, we determined whether the Brd2 interaction with the mutant NS1B F171A protein is also abolished in virus-infected cells. HeLa cells were transfected with the vector encoding the N-terminal Flag tagged Brd2 protein and were infected with wt or F171A virus at a moi of 10 at 24 hours post transfection. Extracts from the infected cells were immunoprecipitated with anti Flag M2 agarose resin and bound proteins were eluted with 3XFlag peptide. Co-purified NS1B proteins were detected by immunoblot analysis using polyclonal anti NS1B antibody. The wild type NS1B protein interacts with Brd2 in cells infected with wt viruses (Figure 3.7 B upper panel lane 7). The non-specifically resin bound NS1B protein in the final eluent was always observed (Figure 3.7 B upper panel lane 8). The amount of the co-purified mutant NS1B F171A protein was only 3-5% of that of the co-purified wild-type NS1B protein (Figure 3.7 B upper panel, compare lanes 7 and 9), demonstrating that the interaction of Brd2 with the NS1B protein in infected cells was almost completely eliminated by the F171A mutation, Taken together with the attenuation of the F171A virus, we can conclude that the Brd2 binding activity of NS1B is required for efficient viral growth of influenza B virus.

Unlike wild type influenza B virus, the F171A virus induces a substantial amount of IFN-β mRNA.

The NS1B effector domain of wild-type influenza B virus inhibits the production of IFN-β. Increased IFN-β synthesis in cells infected with recombinant viruses encoding the N-terminal 104 or 110 amino acids of the NS1B protein was accompanied by a 10- to 100-fold attenuation of virus replication (Dauber et al. 2006; Hai et al. 2008). Similarly, attenuation of the F171A virus may be coupled with the induction of substantial IFN-β. To determine whether this is the case, IFN-β mRNA production was determined by RT-PCR in 293 cells mock infected or infected with either F171A virus or wt virus at 6 and 12 hours post infection (Figure 3.8). Little or no IFN-β mRNA was detected in cells infected by the wt virus (Figure 3.8 A panel 1, lanes 7 to 9), whereas increased IFN-β mRNA was observed in cells infected with the F171A virus (lanes 4 to 6). The same amount of NS1B proteins in cells infected with either virus was detected (Figure 3.8 A panel 3, compare lanes 5, 6 with 8, 9) To determine the quantitative difference in the levels of IFN-β mRNA in cells infected with the wt and F171A viruses, we carried out quantitative real time RT-PCR analysis. The amount of IFN-β mRNA in cells infected with F171A virus was 80 fold more than those in cells infected with wt virus (Figure 3.8 B) at 6 hours post infection. These results indicate the Brd2 binding activity of NS1B is required for suppressing the production of IFN-β mRNA in virus infected cells.

Complexes of IRF3 and CBP are observed in cells infected with F171A virus

The increased IFN-β mRNA in F171A virus-infected cells indicates that the Brd2

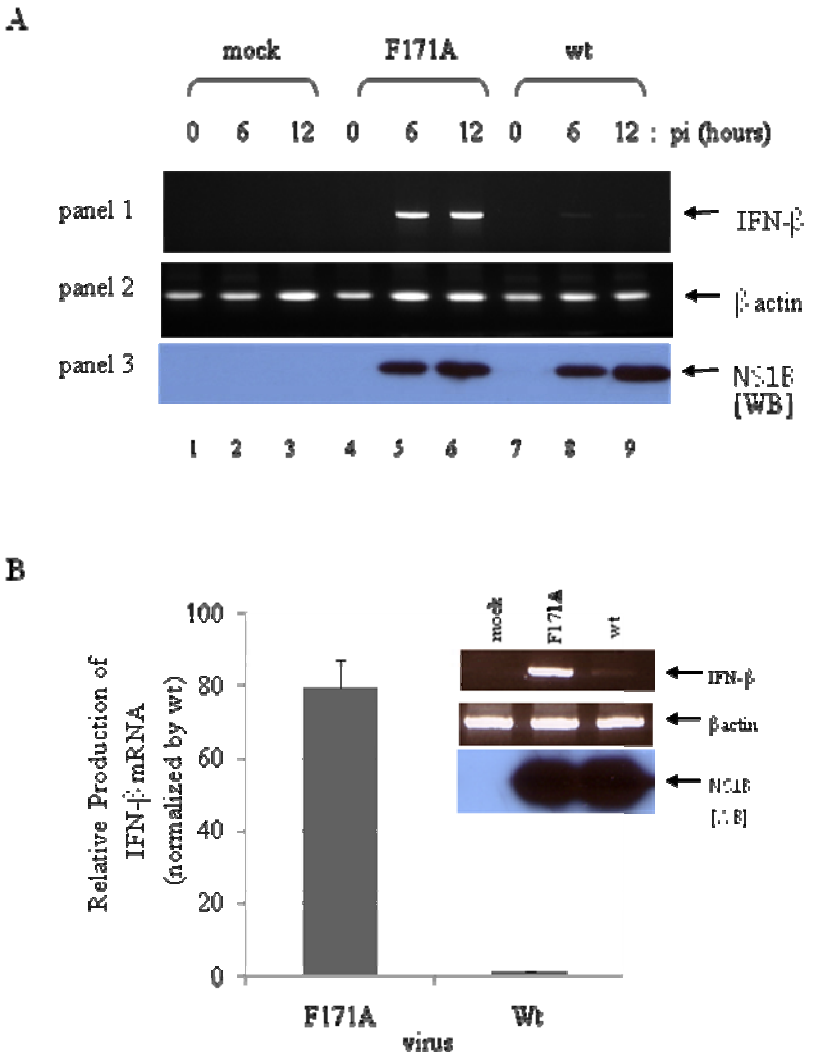

Figure 3.8 More IFN-β mRNA are produced in cells infected with F171A virus. (A) Total RNA was extracted from 293 cells, infected with the viruses, indicated above the panel, at a moi of 3, at 6 and 12 hours post infection (pi) and was reverse transcribed with oligo dT primer. Amplified cDNAs for IFN-β (panel 1) and β actin (panel 2), as a loading control, by PCR were resolved on 0.8% agarose gel. The NS1B protein in extracts from infected cells was detected by Western blotting (panel 3) **(B)** The relative quantity of IFN-β mRNA was determined by quantitative real time RT-PCR as described in Material and Methods. Each bar represents the relative production of IFN-β mRNA in cells infected with either the virus, indicated on X axis. The panels show the result of PCR amplification using same cDNA used for the quantitative real time RT-PCR. As an infection marker, NS1B proteins in extracts, treated in duplicate, were detected by Western blotting. WB: Western blotting

binding activity of NS1B interferes with an upstream step for the transcriptional activation of IFN-β promoter. Moreover, Dauber et al. demonstrated that the NS1B protein inhibits association of IRF3 with CBP, an upstream step for activation of the IFN-β promoter, by detecting the complex between CBP and IRF3 in cells infected with a recombinant virus lacking of the NS1B protein but not in wt virus-infected cells (Dauber et al. 2006). To test if IRF3 can associate with CBP in F171A virus infected cells, endogenous CBP was co-immunoprecipitated with the N-terminal Flag tagged IRF3 protein. A large amount of a CBP-IRF3 complex was observed in F171A virus–infected cells (Figure 3.9, lane 9), whereas only 1% as much of this complex was detected in wt virus-infected cells on darker exposure of gel (lane 11). A similar low amount of this complex was detected in extracts from the mock-infected cells on darker exposure of gel (lane 7).

IRF3 is activated in cells infected with F171A virus.

The cytoplasmic latent IRF3 is activated by virus infection and activated IRF3 dimerizes, followed by their nuclear accumulation to complex with CBP/p300. Therefore, IRF3 dimerization is the upstream step of the association of CBP and IRF3, previously tested. To test if IRF3 is activated in cells infected with F171A virus, we performed the IRF3 dimerization assay. Influenza A/Udorn/72 virus (Udorn virus) (Das et al. 2008) and a recombinant influenza B virus encoding the N-terminal 104 amino acids of NS1B protein (104 virus) were used as positive control. Two human cells, HEK 293 cells and HEL299 cells were used as host cell in this assay. HEK293 cell is a human fibroblast cell.

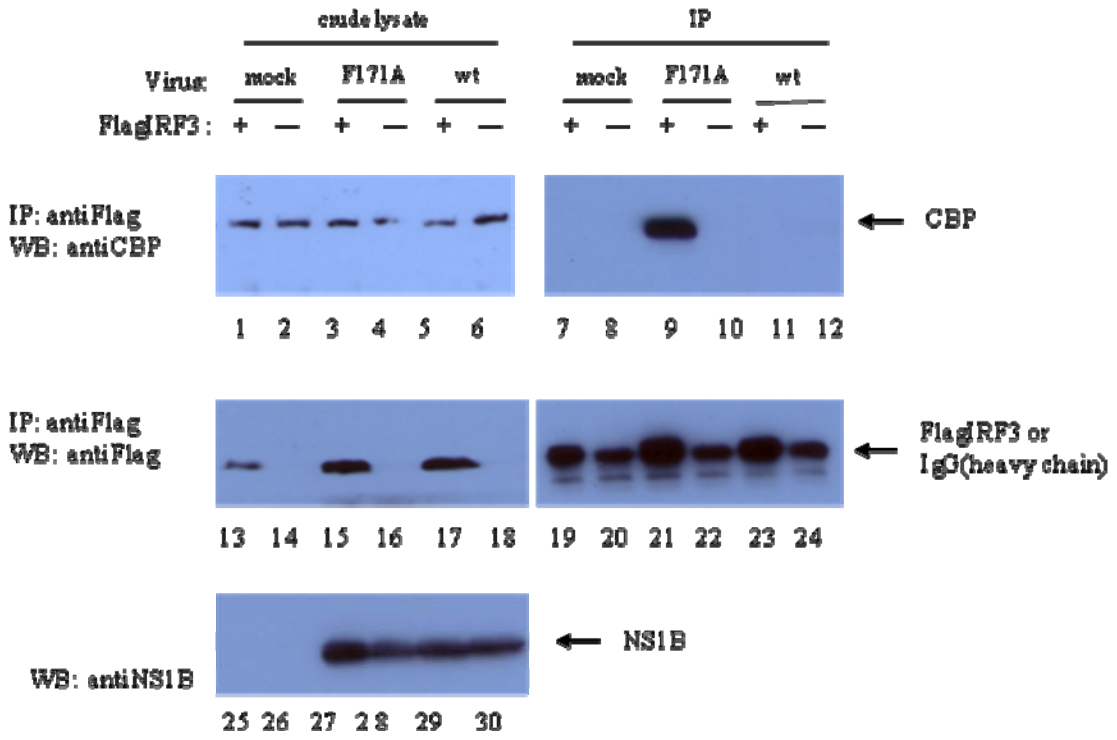

Figure 3.9 Increased associations of CBP and IRF3 are observed in cells infected with F171A virus than in those infected with wt virus. Subconfluent 293 cells on 10cm plate, transfected with 10μg of plasmid encoding the N-terminal Flag tagged IRF3, were infected with the viruses indicated above at a moi of 5 at 24 hours post transfection. Extracts, prepared at 8 hours post infection, were immunoprecipitated with anti Flag M2 resin. The purified (lanes 19 to 24) and co-purified proteins (lanes 7 to 12) were detected by Western blotting using antibodies as needed. The FlagIRF3 (lanes 13 to 18), CBP, (lanes 1 to 6), and NS1B proteins (lanes 25 to 30) in crude lysate were detected by Western blotting.

HEL299 is a lung cell line, a major site of influenza virus infection. IRF3 dimers were detected in both cell lines infected with F171A viruses (Figure 3.10 A and B, lane 4), similar to the dimerization observed in cells infected with the Udorn and 104 viruses (lanes 2 and 3). Little or no IRF3 dimers were detected in cells infected with the wt influenza B virus (lane 5). Expression patterns of NS1 proteins in HEK 293 cells or in HEL299 cells are different. All influenza B viruses in 293 cells expressed similar amounts of NS1B proteins (Figure 3.10 A third panel from top, lanes 2 to 5), but the amount of the mutant NS1B proteins in HEL299 cells infected with the mutant influenza B viruses was less than that in cells infected with wt viruses (Figure 3.10 B third panel from top). In HEL299 cells the 104 virus expressed a substantially lower amount of the truncated NS1B protein (compare lanes 3 with 5), and the F171A virus expressed less NS1B protein than the wt virus (compare lanes 4 with 5). These results probably indicate that the 104 and F171A viruses are more attenuated in HEL299 cells than in 293 cells. The results indicate that the Brd2 binding to the NS1B effector domain suppresses IRF3 activation and dimerization in virus-infected cells.

The Brd2 binding activity of the NS1B effector domain specifically targets at or near TBK1.

MAVS and TBK1 are the upstream factors in the pathway for the induction of IFN-β gene in virus-infected cells (Chapter one, Figure 1.9). To determine which factor(s) is inhibited by the Brd2 binding activity of the NS1B effector domain, we

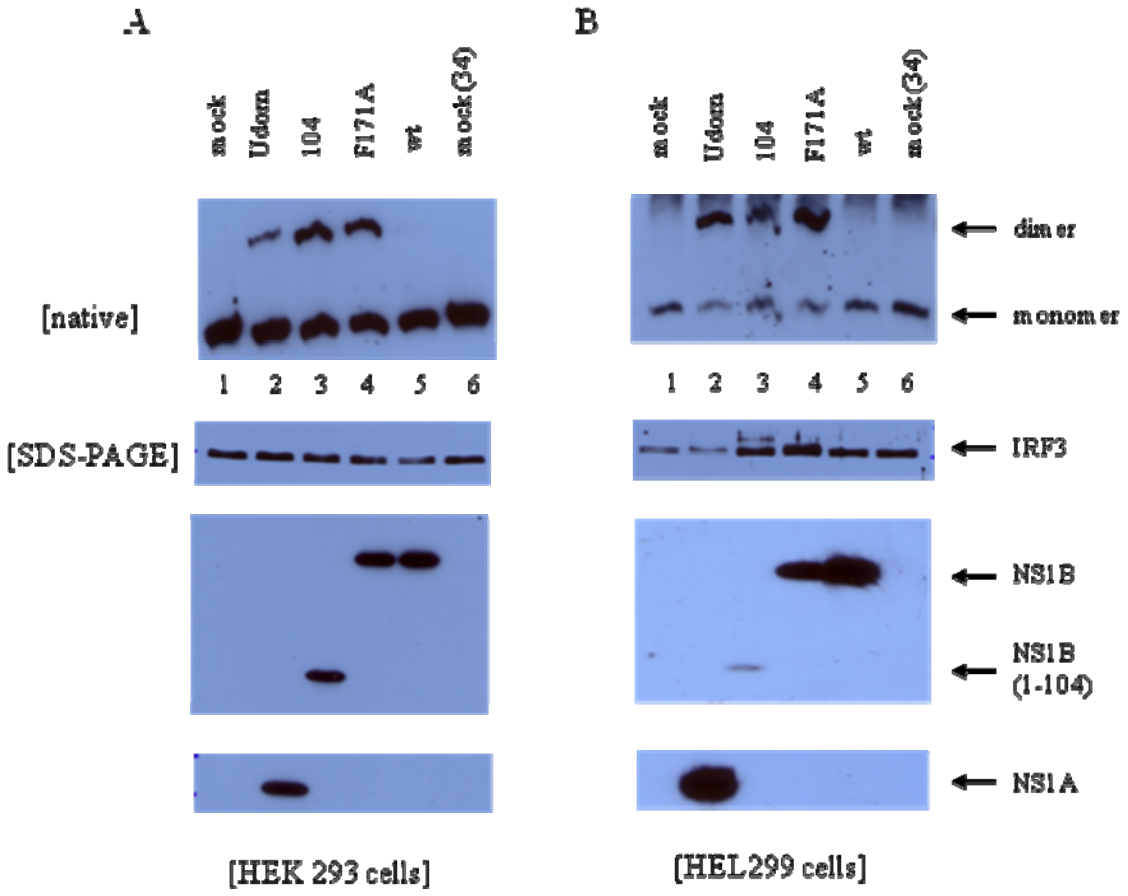

Figure 3.10 IRF3 is activated in human cells infected with F171A virus. Extracts from HEK293 cells **(A)** or HEL299 cells **(B)**, infected with influenza A/Udorn/72 (Udorn) (lane 2) or one of influenza B viruses including wild type (Wt) (lane 5) and mutants (F171A virus and 104 virus) (lanes 3 and 4) at a moi of 1, were prepared at 6 hours post infection. The extracts were resolved by Native- or SDS-PAGE. IRF3 dimers, resolved by Native-PAGE, were detected by Western blotting using polyclonal anti IRF3 antibody. Total IRF3 in extracts, subjected to SDS-PAGE, were detected by Western blotting using same antibody. The NS1 proteins in extracts were detected by immunoblot analysis using polyclonal rabbit anti NS1A or NS1B antibody.

carried out transfection assays in 293T cells to compare the effects of the wt and F171A mutant NS1B effector domains on the ability of the following factors to activate the IFN-β promoter encoding luciferase; MAVS or TBK1 (Figure 3.11A). Both effector domains exhibited partial inhibition of the MAVS activation of the IFN-β promoter, with the wt effector domain exhibiting more inhibition than the F171A effector domain (60% versus 40%) (lanes 4 to 6). The most dramatic results were observed with the TBK1 activation of the IFN-β promoter: the wt effector domain inhibited the activation almost completely, whereas the F171A effector domain inhibited activation only about 10% (lanes 7 to 9). Consequently, the F171A mutation that eliminated Brd2 binding essentially eliminated the ability of the NS1B effector domain to inhibit TBK1 activation of the IFN-β promoter. As confirmation, we showed that the inhibition of the TBK1-mediated IFN-β promoter activation by the wt effector domain increased with increasing amounts of the plasmid expressing this effector domain, whereas little or no inhibition by the F171A effector domain occurred even with the highest amount of transfected plasmid (Figure 3.11B). These results indicate that the Brd2 binding activity of the NS1B effector domain likely targets at or near the step in IRF3 activation mediated by the TBK1 factor.

siRNA knockdown experiments

As documented above, the F171A mutation renders the NS1B effector domain inactive in suppressing IRF3 activation. The increased IFN-β in mutant virus-infected cells results in the attenuation of the replication of F171A virus. To verify that the loss of Brd2 binding by the F171A effector domain is responsible for the activation of IRF3 and

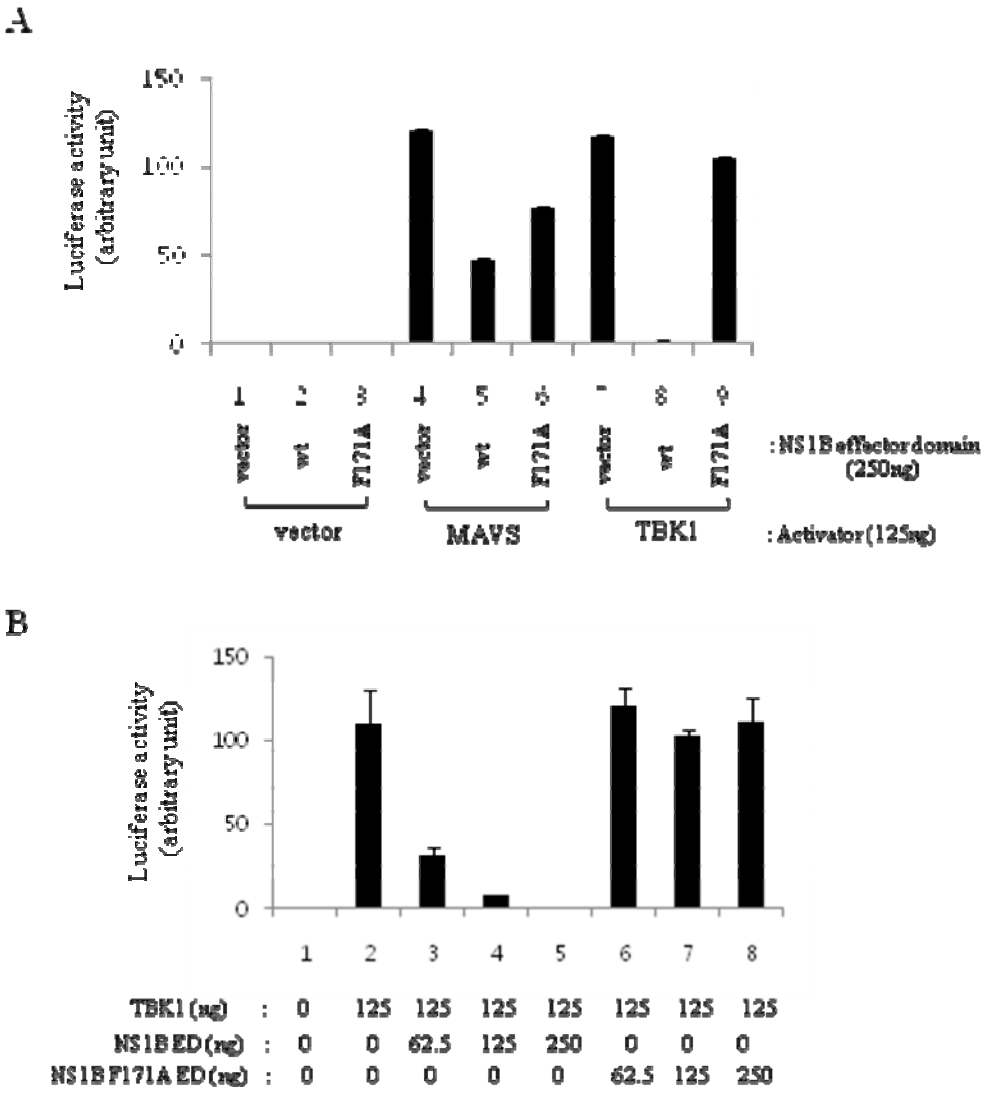

Figure 3.11 The Brd2 binding activity to the NS1B effector domain targets at or near TBK1 and the effector domain inhibits the TBK1-activation of the IFN-β promoter dose-dependently. (A), (B) 293T cells, on 24-well plate, were transfected with the indicated amounts of plasmids encoding the proteins, designated on X-axis, together the mixture of plasmids encoding fire fly luciferase under the control of IFN-β promoter (pIFNβpL, 100ng) or renilla luciferase as an internal control (pRL, 25ng). Luciferase activities in extracts from the transfected cells were measured using luminometer at 48 hours post transfection. All experiments were performed in duplicate and all fire fly luciferase activities were normalized to those of renilla luciferase. NS1B ED: the NS1B effector domain; NS1B F171A ED: the NS1B F171A effector domain.

IFN-β transcription, we carried out siRNA experiments to deplete the Brd2 protein from cells infected by the wt and F171A viruses. We used HEK293 cells and HEL299 cells for these experiments because we already demonstrated the F171A virus-infection dependent IRF3 activation (Figure 3.10 A and B).

The depletion of Brd2 in HEL299 cells was extremely inefficient (data not shown), and approximately 50% of the endogenous Brd2 protein was depleted in HEK293 cells at 72 hours post transfection of 100nM Brd2 siRNA (siBrd2) (Figure 3.12 A). To determine whether this incomplete depletion of Brd2 was sufficient to observe an effect, the siRNA-transfected HEK 293 cells were infected with the wt or F171A virus at a moi of 3 for 6 hours, and IRF3 dimers in the infected cells were detected by IRF3 dimerization analysis (Figure 3.12 B). IRF3 was activated in cells infected with the F171A virus (Figure 3.12 B, lanes 2 and 5), showing that the 50% suppression of total endogenous Brd2 protein did not make any difference in the amount of IRF3 dimers (Figure 3.12 B, compare lanes 2 with 5). IRF3 in wt virus- or mock- infected cells was not activated (Figure 3.12 B, lanes 1, 3, 4, and 6). The results suggest that the remaining half of the cellular Brd2 is sufficient for suppressing the activation of IRF3 in wild type influenza B virus-infected cells, emphasizing the need for more efficient depletion of endogenous Brd2.

To attempt more efficient knockdown of Brd2, we employed a plasmid encoding short-hairpin RNA (shRNA). In addition, to assay for partial as well as complete reversal of the F171A phenotype, we measured the levels of IFN-β mRNA using RT-PCR. We generated 11 plasmids encoding shRNAs against the Brd2 mRNA. Plasmid #11

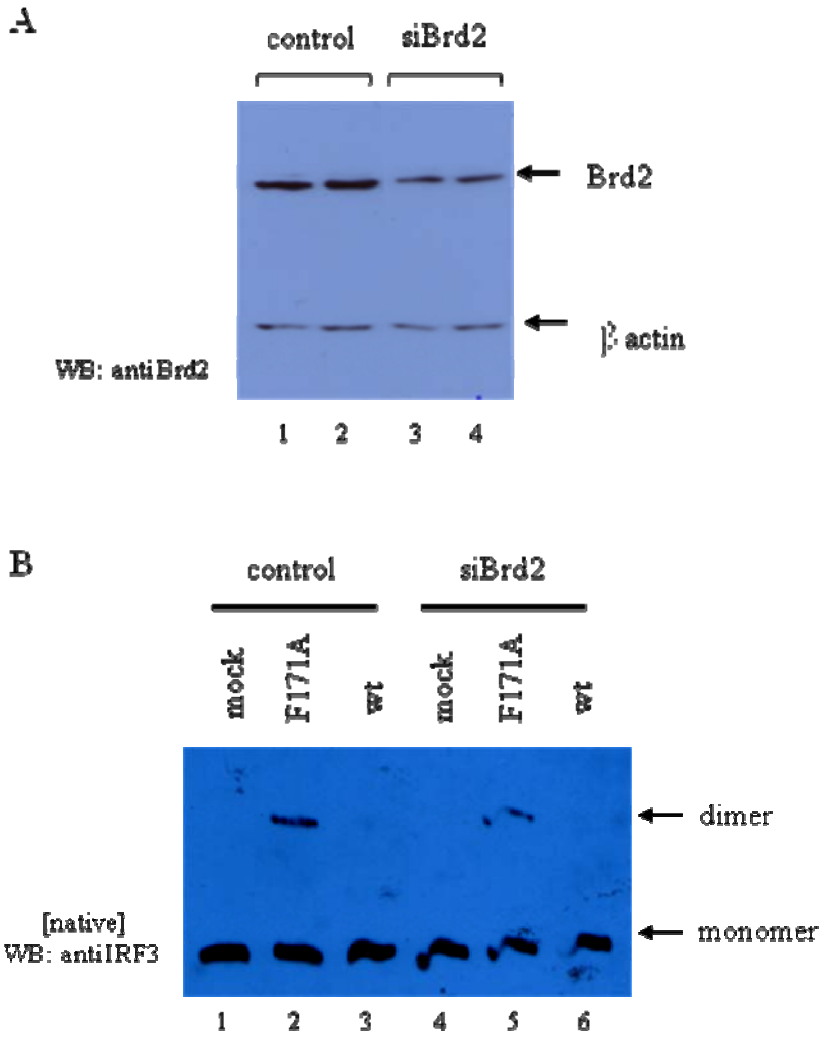

Figure 3.12 50% of total endogenous Brd2 proteins in HEK293 cells are depleted by siRNA transfection and the knock down efficiency is not sufficient. (A) HEK293 cells were transfected with 100nM of control or siBrd2 using Dharmafect I as a transfection reagent. Extracts from the transfected cells were prepared at 72 hours post transfection. The Brd2 proteins in the cells were detected by Western blot analysis and the β actin proteins on same blot were detected with anti β actin antibody. **(B)** Extracts from HEK293 cells, transfected siRNA as described in (A) and infected with the indicated viruses at a moi of 3, were subjected to IRF3 dimerization assay at 6 hours post infection.

(shBrd2 #11) was chosen because it depleted 90% of endogenous Brd2 proteins in 293 cells were equally transfected with plasmid encoding shBrd2 #11. As a control, each set of cells were transfected with plasmid encoding control shRNA (control) or not transfected with any plasmid (w/o shDNA). The cells were infected with the wt or F171A virus at 72 hours post transfection. RNA was extracted from one set to detect IFN-β mRNA, and extracts from the other set were subjected to Western blot analysis at 6 hours post infection. Ninety percent of the cellular Brd2 protein was depleted in cells transfected with the shBrd2 #11 (Figure 3.13 panel 1 lanes 4 to 6). However, IFN-β mRNA was nonspecifically induced in cells transfected with plasmid encoding either the control or Brd2-specific shRNA (Figure 3.13 panel 4, lanes 1 to 6). The increased IFN-β production significantly interfered with viral replication, resulting in the decreased expression of the NS1B protein (Figure 3.13 panel 3, compare lanes 2, 3, 5, and 6 with 8 and 9). These results suggest that the shRNA approach, even using cells containing retrovirus vectors constitutively expressing Brd2-specific shRNAs, will not succeed.

The NS1B effector domain also binds another member of the Brd family proteins, Brd4

We decided to determine whether there was a further complication, i. e., whether other members of the Brd family protein also bind to the NS1B protein, even though they were not detected in the mass spectrometric analysis. To test this possibility, we determined whether the NS1B protein interacted with two other Brd family proteins, Brd3 and Brd4, using the GST pull down assay. The Brd3 and Brd4 proteins contained an

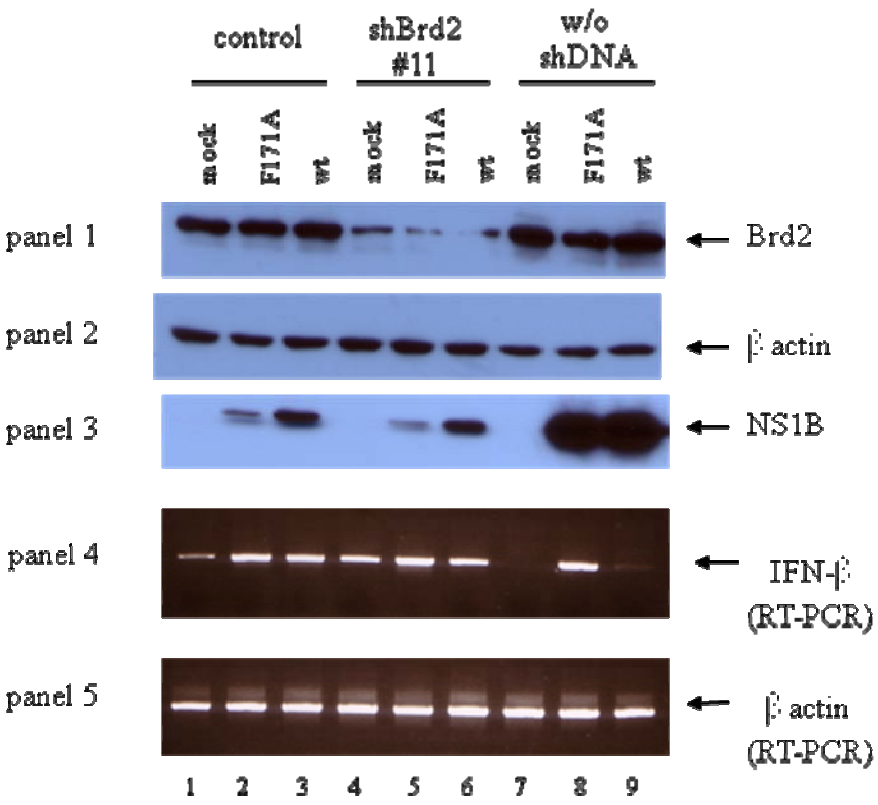

Figure 3.13 90% of total Brd2 proteins in HEK293 cells are depleted by transfecting plasmid encoding shBrd2 #11 but the shDNA induces the synthesis of IFN-β mRNA nonspecifically. HEK293 cells were transfected with plasmid encoding control shRNA (control), Brd2 specific shRNA (shBrd2), or not transfected with any plasmid (w/o shDNA). The transfected cells were infected with viruses as indicated at a moi of 3 at 72 hours post transfection. Total RNA was extracted from the infected cells and IFN-β mRNA was detected by RT-PCR (panel 4). The β actin mRNA was detected as loading control (panel 5). Extracts from the infected cells, treated in duplicate, were subjected to Western blot analysis (panels 1 to 3).

N-terminal Flag tag, and the NS1B protein contained N-terminal GST (Figure 3.14). Brd3 did not interact with the NS1B protein (panel 1, lane 7), whereas Brd4 did interact, although about 5%, as well as Brd2 (panel 1, compare lanes 6 and 8). Brd4 as well as Brd2 lost its interaction capability with mutant NS1B F171A protein (data not shown). Consequently, Brd4 might be able to compensate for depletion of Brd2.

DISCUSSION

The inhibition of IFN-β production by the NS1B effector domain in influenza B virus-infected cells has been documented (Dauber et al. 2006; Hai et al. 2008), but the mechanism of this inhibition has not been elucidated. We have provided evidence that this inhibition is mediated at least in part by the binding of the cellular Brd2 protein to the NS1B effector domain. We identified Brd2 as a protein that interacts with the NS1B effector domain, and showed that the F at position 171 of the NS1B protein is essential for Brd2 binding. A recombinant influenza B virus encoding a NS1B protein with the F171A mutation is attenuated, coupled with the activation of IRF3 and the induction of a large (80-fold) increase in IFN-β mRNA in infected cells. Transfection assays implicated the activation of the TBK1 kinase as the step in IRF3 activation that is induced in F171A mutant virus-infected cells. However, attempts at further confirmation by depletion of endogenous Brd2 using RNA interference were not successful because of inefficient knock-down efficiency and nonspecific IFN-β induction.

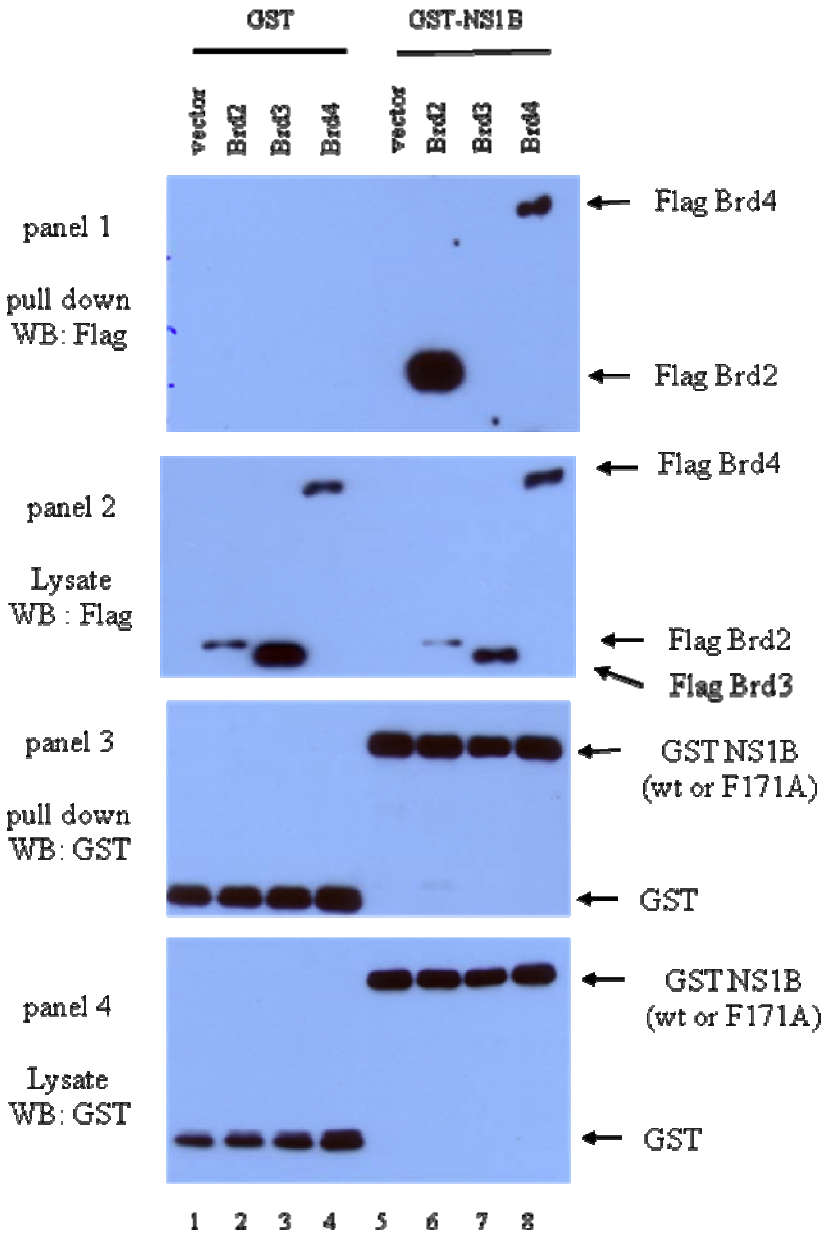

Figure 3.14 Brd4 interacts with the NS1B protein but Brd3 does not. 293T cells were co-transfected with a plasmid encoding GST or the N-terminal GST fused NS1B protein together a plasmid encoding one of the N-terminal Flag tagged Brd2, 3, and 4 proteins (FlagBrd2, FlagBrd3, and FlagBrd4). Extracts from the transfected cells were selected on glutathione sepharose affinity resin and the selected GST or GST fused NS1B protein (panel 3) and the co-selected Flag tagged Brd proteins (panel 1)were detected by immune blotting analysis. The proteins in extracts were detected Western blot analysis (panel 2 and panel 4).

Our current model for the way that Brd2 interferes with the activation of IRF3 is shown in Figure 3.15. We propose that Brd2 interferes with the activity of the TBK1 kinase which is responsible for phosphorylating IRF3 via binding to the NS1B protein. Another viral protein, the K7 protein of vaccinia virus, interferes with the activation of TBK1. The K7 protein does not directly bind to TBK1, but rather binds to DDX3, a DEAD box helicase, which transiently binds to TBK1 and enhances its activity (Schroder et al. 2008). The K7 protein binds to the N-terminal region of DDX3 that is required for activation of TBK1, suggesting that the K7 protein competes with TBK1 for a binding site in DDX3 in vaccinia virus-infected cells. As another TBK1-targeting mechanism, the $\gamma_1$34.5 protein, a Herpes simplex viral protein, forms complex with TBK1 and disrupts the interaction of TBK1 and IRF3. Consequently, the disruption caused the inhibition of TBK1 mediated IRF3 activation (Verpooten et al. 2009). Our future research will determine whether the active form TBK1, which is phosphorylated its serine residue at position 172 (Kishore et al. 2002), is increased in cells infected by the F171A virus. We have already found that the NS1B protein in wt virus-infected cells does not bind TBK1 (data not shown).

All previous studies of the Brd2 protein focused on its functions in the nucleus. Brd2 transactivates the promoters of several cell cycle regulatory factors through binding to the E2F transcription factor (Denis et al. 2000; Sinha et al. 2005), and it also facilitates the transcriptions of several genes by binding to hyperacetylated chromatin (LeRoy et al. 2008). Interestingly, the localization of the Brd2 protein is dynamic. In mouse cells, Brd2 in proliferating cells is found predominately in the nucleus, but localizes to the cytoplasm

in cells as they exit the cell cycle (Crowley et al. 2002; Crowley et al. 2004). Brd2 is spread out throughout cells that are serum starved, but mitogen stimulation of such cells causes nuclear localization of the Brd2 protein (Guo et al. 2000). The function of the cytoplasmic Brd2 in resting cells has never been studied. The localization of the NS1B protein in infected cells is also dynamic. The NS1B protein localizes mainly in nuclear speckles at early times of infection, but moves to the cytoplasm at later times of infection (Schneider et al. 2009). Because of this pattern of NS1B protein localization, it is important to determine the localization of the Brd2 protein at various times after infection with the wt and F171A viruses and compare this to the time course of activation of IRF3 and induction of IFN-β mRNA synthesis. To address this issue, we will check the localization of the Brd2 protein in wt and F171A virus-infected cells using immunofluorescence microscopy analysis at various hours post infection.

The Brd4 protein, another member of the Brd family, also interacts with the NS1B protein, although less efficiently than Brd2. This finding further complicates the use of siRNA knockdown experiments to confirm the role of Brd2 binding to NS1B in the inhibition of IRF3 activation. Even if we overcame the difficulties described above in achieving specific and efficient depletion of the Brd2 protein, the Brd4 protein would probably compensate for the loss of the Brd2 protein. Double depletion of both Brd2 and Brd4 is probably not feasible, because it would arrest the cell cycle at the G1 phase (Mochizuki et al. 2008). As another approach, we are identifying the NS1B binding domain on Brd2, which is probably also on Brd4, and will determine whether this domain has a dominant negative effect in the suppression pathway in virus-infected cells.

In summary, to elucidate the mechanism by which the effector domain of the NS1B protein suppresses IFN-β production in influenza B virus-infected cells, we screened for a cellular protein that interacts with the NS1B effector domain, and identified Brd2 as such an interacting protein. We have provided evidence that Brd2 binding to the NS1B protein mediates the suppression of IRF3 activation and the synthesis of IFN-β mRNA in virus-infected cells. However, as already discussed, further confirmatory results are still needed.

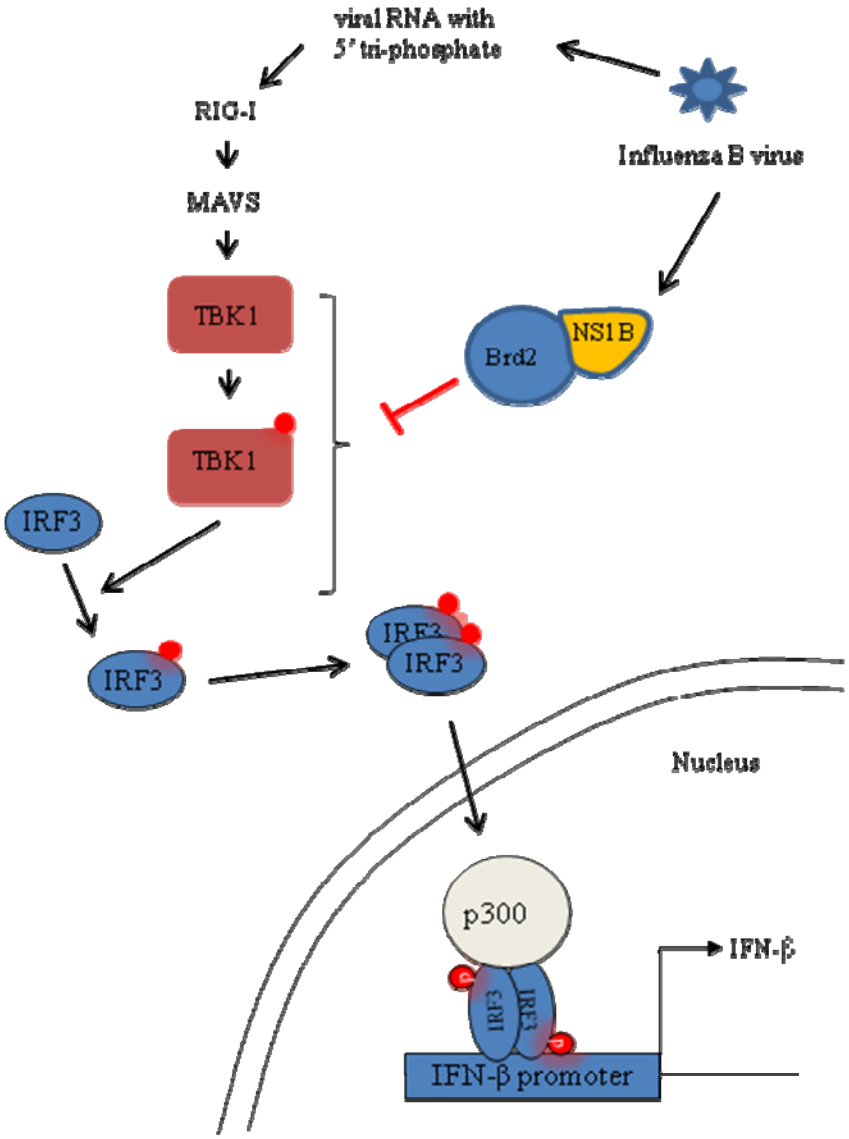

Figure 3.15 Working model Brd2 might interfere with the activation of IRF3 by targeting at or near TBK1 through binding to the NS1B protein in virus-infected cells.

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